University of Portsmouth

The Role of Coagulation Factors in the Regulation of Bronchial Epithelial Repair *In Vitro*

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Abstract

The bronchial epithelium serves as a protective cellular barrier and the first site of contact for damaging inflammatory and physical stimuli from the environment. Previous *in vivo* studies in guinea pigs have demonstrated that normal bronchial epithelial repair is both rapid, and dependent on the formation of a provisional fibrin matrix that is exclusively plasma-derived. This study tests the hypothesis that local release of coagulation factors from bronchial epithelial cells and subsequent fibrin formation supports bronchial epithelial repair *independently* of plasma proteins.

Cells of the 16HBE 14o$^-$ cell line and primary normal human bronchial epithelial (NHBE) cells were cultured in serum-free medium to confluence and mechanically wounded. The expression of coagulation factors in response to wounding was examined at the protein and mRNA level using immunohistochemistry, immunoblot, western blot and RT-PCR. The role of coagulation factors in epithelial repair was investigated using neutralising antibodies and specific inhibitors and monitored by both standard light and kinetic video microscopy. The role of PARs in fibrin formation and epithelial repair was investigated using specific PAR-1 and PAR-2 peptide agonists.

Active TF was constitutively expressed in 16HBE 14o$^-$ cells and initiated the coagulation cascade that was essential for epithelial repair. Fibrinogen and FXIII were rapidly released, within 20 minutes in response to wounding and were essential for wound repair. Fibrin formation and turnover was demonstrated by the release of D-dimers from epithelial cell layers in proportion to the degree of wounding. Expression of tissue factor (TF) and factor VII (FVII) mRNA increased *post*-wounding over an extended time course up to 12 hours.

The bronchial epithelium is therefore a source of preformed coagulation factors released rapidly in response to mechanical wounding and the autonomous formation of a cross-linked fibrin matrix is essential for wound repair.

In asthma, the bronchial epithelium has increased susceptibility to injury and normal repair mechanisms are compromised, contributing to bronchial hyper-reactivity. The data presented in this thesis suggests novel therapeutic strategies to enhance repair of the damaged bronchial epithelium in asthma, such as the delivery of recombinant FXIII by inhalation or the use of specific agonists of PAR-2, which is associated with cytoprotection in the lung.

Publications

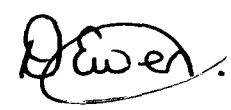
Ewen, D., Vayro, S., Trevethick, M., Salmon, G. and Shute, J.K. (2005). Tissue Factor plays a central role in bronchial epithelial repair responses. *Eur. Res. J*, **26**, Supplement 49; P3579.

Ewen, D., Trevethick, M., Salmon, G. and Shute, J.K. (2005). A role for PARs in bronchial epithelial repair. *Eur. Res. J*, **26**, Supplement 49; P3584.

Perrio, M. J., Ewen, D., Trevethick, M., Salmon, G. and Shute, K. (2007). Fibrin formation by wounded bronchial epithelial cell layers *in vitro* is essential for normal epithelial repair and independent of plasma proteins. *Clin. Exp. Aller,* **11**; P1688-1700.

Declaration

I declare that while registered as a candidate for the degree of Doctor of Philosophy of the University of Portsmouth, I have not been registered as a candidate at any other university.



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ACh	Acetylcholine
ADAM	A disintegrin and metalloproteinase
AHR	Airway hyperresponsiveness
ALI	Acute lung injury
ALI	Air liquid interface
AP-1	Activator protein-1
APC	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
Arg	Arginine
ASM	Airway smooth muscle
Asp	Aspartic acid
ATP	Adenosine triphosphate
AU	Arbitrary units
BAL	Bronchoalveolar lavage
BEGM	Bronchial epithelial growth medium
bp	Base pair
BSA	Bovine serum albumin
cAMP	Adenosine 3′, 5′- cyclic monophosphate
CBF	Ciliary beating frequency
cDNA	Complementary DNA
CF	Cystic fibrosis
Cfegs	Clusters of free eosinophil granules
cGMP	Guanosine 3′, 5′- cyclic monophosphate
cNOS	Constitutive nitric oxide synthase
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRTH2	Chemoattractant receptor of Th2 cells
CSLM	Confocal scanning laser microscopy
cys-LTs	Cysteinyl-leukotrienes
$CysLT_1$	Cysteinyl leukotriene receptor type 1
DEP	Diesel exhaust particles
Der P	*Dermatophagoides pteronissinus*

DMSO	Dimethylsulphoxide
dNTP	d-Nucleoside triphosphate
DSS	Disuccinimidyl suberate
DTSSP	3,3´-Dithiobis(sulfosuccinimidylpropionate)
DTT	Dithiothreitol
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbant assay
EMTU	Epithelial mesenchymal trophic unit
eNOS	Endothelial nitric oxide synthase
EP	E-prostanoid
EPa	Elastolytic metalloproteinase
EPO	Eosinophil peroxidase
EPR	Effector cell protease receptor
FBS	Foetal bovine serum
FGC	Fibrinogen gamma chain
FGF	Fibroblast growth factor
FIX	Factor IX
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FTRIFD	Protease activated receptor-1 control peptide agonist
FVa	Activated factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIII	Factor XIII
FXIIIa	Activated FXIII
FXIIIA	Factor XIII A subunit
Gla	Gamma-carboxyglutamic acid-rich

GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptor
GTP	Guanosine triphosphate
HAEC	Human airway epithelial cells
HAT	Histone acetyl transferase
HB-EGF	Heparin-binding epidermal growth factor
HBSS	Hanks' balanced salt solution
HDAC	Histone deacetylase
HDACI	Histone deacetylase inhibitor
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HETE	Hydroxyeicosatetraenoic acid
HGF	Hepatocyte growth factor
His	Histidine
HMG	High mobility group
hr	Human recombinant
HRP	Horseradish peroxidase
HSAE	Human small airway epithelial
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ITS	Insulin-transferrin-sodium selenite
JNK	*c*-Jun kinase
KGF	Keratinocyte growth factor
KGFR	Keratinocyte growth factor receptor
LAP	Latency associated peptide
LDH	Lactate dehydrogenase
Leu	Leucine
LPS	Lipopolysaccharide
LSIGKVD	Protease activated receptor-2 control peptide agonist
LT	Leukotriene
mAb	Monoclonal antibody

MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCP	Monocyte chemoattractant protein
MDC	Monocyte-derived chemokine
MEM	Minimal essential medium
MMC	Mitomycin C
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NHBE	Normal human bronchial epithelial
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSB	Non-specific binding
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PD-031	FXa inhibitor
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDI	Protein disulphide isomerase
PF4	Platelet factor 4
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PKA	Protein kinase A
Pro	Proline
RANTES	Regulated upon activation normal T cell expressed and secreted

RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecylsulphate
SDS PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
Ser	Serine
SLPI	Secretory leukocyte peptidase inhibitor
TAFI	Thrombin activatable fibrinolysis inhibitor
TARC	Thymus and activation regulated chemokine
TBP	TATA box-binding protein
TBS	Tris buffered saline
TF	Tissue factor
TFF	Trefoil factor family
TFPI	Tissue factor pathway inhibitor
TFRIFD	Protease activated receptor-1 peptide agonist
TGF	Transforming growth factor
Th2	T-helper 2
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF	Tumour necrosis factor
t-PA	Tissue-type plasminogen activator
TSA	Trichostatin A
TXA_2	Thromboxane A_2
scu-PA	Single chain urokinase-type plasminogen activator
SLIGKVD	Protease activated receptor-2 peptide agonist
UK-156,406	Thrombin inhibitor
UK-220,047	FXa inhibitor
u-PA	Urokinase-type plasminogen activator
u-PAR	Urokinase-type plasminogen activator receptor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VLA	Very late antigen
VWF	Von Willebrand factor
ZO	Zonula occludens
5-LO	5-lipoxygenase

Chapter 1.

General Introduction

1. General Introduction

1.1. The bronchial epithelium

The bronchial epithelium is primarily a protective cellular barrier, which acts as a first site of contact for inflammatory and physical stimuli from the environment (Holgate *et al.*, 2000). The bronchial epithelium is comprised of specialised epithelial cells and the integrity of the epithelial layer is dependent upon various mechanisms of adhesion (Montefort *et al.* 1992). Irrespective of whether the phenotype is normal or asthmatic, the bronchial epithelium is continually subjected to inhaled pollutants, proteolytic allergens and microbes and is frequently injured as a consequence. An intact bronchial epithelium is vital for normal airway function; therefore the repair response must be rapid to restore the damaged epithelium following injury. The normal bronchial epithelium has exceptional ability to repair itself following injury.

1.1.1. Structural composition of the bronchial epithelium as a protective barrier

The structure of the airway epithelium has been extensively studied and it has been shown that at least eight distinct types of epithelial cell are present in the human respiratory tract (Jeffery, 1983). These may be classified into three main categories of cell type described as basal, ciliated and secretory.

The predominant cell type within the airways accounting for over half of all epithelial cells is the columnar ciliated epithelial cell, which arises from either the basal or secretory cell (Knight *et al.*, 2003). The important feature of the columnar ciliated cell is that it bears up to 300 cilia per cell on the apical surface, allowing it to perform its unique role of mucus transportation from the lung to the throat in order to be expelled.

Basal cells are extensively present throughout the bronchial epithelium. In addition to their ability to differentiate into mucous and ciliated epithelial cells, they play a significant intermediary role in the binding of the columnar epithelial cells to the underlying basement membrane (Evans *et al.*, 1990). This is made possible by the characteristic of basal cells to express hemidesmosomes, which in turn, possess the

necessary integrins ($\alpha 6\beta 4$) to attach to the basement membrane. Basal cells are also thought to be involved in the secretion of a number of bioactive molecules, including neutral endopeptidase, 15-lipoxygenase products and cytokines (Knight and Holgate, 2003).

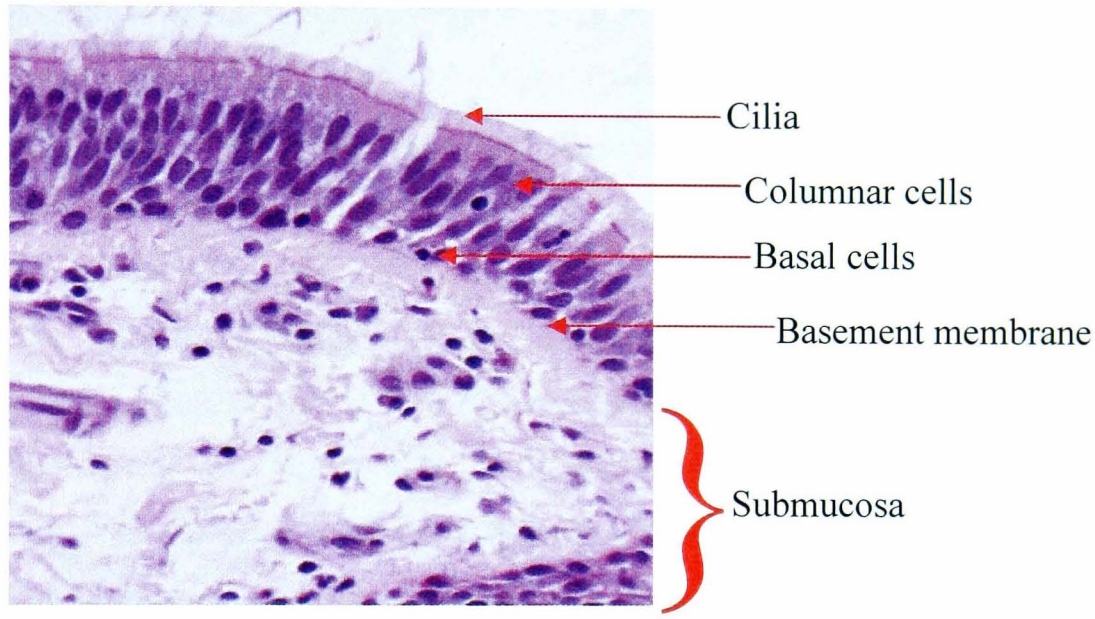

Figure 1.1. Histological section through the bronchial epithelium of a non-asthmatic subject. The intact, normal bronchial epithelium is pseudostratified, with columnar cells, situated on top of basal cells, attached to a basal membrane.

Of the eight epithelial cell types within the bronchial epithelium, the goblet, serous and clara cells comprise the three secretory cells (Reid *et al.*, 1980). The mucus lining of the respiratory tract originates from products of secretory cells interspersed among mucosal cells or within submucosal glands and protects the underlying mucosa from dehydration. Current understanding is that the lining is a two-fluid model in which the upper layer is a viscoelastic gel (mucous, cross-linked glycoproteins) that overlies a sol layer (serous). Thus, mucous propelled by ciliary beating flows above the sol layer and contains sloughed cells and xenobiotic materials that come into contact with it (Foster, 2002). Goblet cells are highly abundant in the normal human airways. They produce mucous and are also involved in mucociliary clearance. Goblet cells contain acidic-mucin granules, which swell to form mucin granules when released, in order to capture foreign particulates in the airway lumen (Jeffery, 1983). In addition to mucous production by these cells, the acidity of the mucin granules is also vital to the transportation of mucous across membranes into the airway lumen. The acidity of these mucin granules is substantially increased in response to exposure of harmful agents such as sulphur dioxide or cigarette smoke (Knight and Holgate, 2003). Goblet cells are

adaptive since they are capable of self-renewal and of differentiation into ciliated epithelial cells (Evans *et al.*, 1988).

Serous cells are structurally similar to goblet cells and share a role in mucociliary clearance. Until fairly recently, serous cells were only identified in the rat airway (Rogers *et al.*, 1993). Little is known about these cells in the human trachea, however, it is well established that, in the rat airway they contain neutral mucin granules and an unidentified non-mucoid substance.

Clara cells are present in both the bronchial and bronchiolar airways. They contain electron-dense granules, which in turn are considered to contain bronchiolar surfactant. In addition, they are believed to be involved in the metabolism of xenobiotic compounds by the action of p450 mono-oxygenases and are also speculated to produce specific antiproteases (Knight and Holgate, 2003). Clara cells are believed to play an important role as a progenitor for the ciliated columnar or mucous-secreting cells (Hong *et al.*, 2001).

1.1.2. Maintenance of the bronchial epithelium

The three major components involved in the maintenance of a protective barrier consist of: desmosomes, zonula adherens (intermediate junction) and tight junctions (Rennard *et al.*, 1991). Of these junctional complexes, desmosomes are a major structural adhesion mechanism and appear to be the most prominent.

Desmosomes are symmetrical structures, which are composed largely of plaques and anchoring tonofilaments and serve as junctional 'spot-welds' between cells (Montefort *et al.*, 1992). They are located between the basal cells and the differentiated cells, hence the reason that they have been associated with the regulation of cellular injury and detachment. Hemidesmosomes are responsible for anchoring the basal cells to the underlying basement membrane in order to maintain the bronchial epithelium. The few columnar cells that penetrate down to the basement membrane fail to form hemidesmosomal links with the basement membrane and are therefore dependent on their attachment to basal cells by desmosomes for their anchorage (Evans *et al.*, 1989; Evans *et al.*, 1988). In a study involving the analysis of bronchoalveolar lavage (BAL)

fluid from asthmatic subjects, Montefort *et al* (1992) concluded that the plane of cleavage lies between the columnar and basal cell layers following epithelial damage, since the BAL samples consisted almost entirely of columnar cells with very few basal cells present (Montefort *et al.*, 1992).

The intermediate junctions form a ring-like adhesive mechanism around the cell and resemble desmosomes with the exception of possessing a narrower intercellular space with cytoplasmic filaments in place of the dense plaque, which is present in desmosomes. Little information is known about the composition of these structures; however, early studies demonstrated the presence of the cadherin, uvomorulin at this junction (Boller *et al.*, 1985). Cadherins are calcium-dependent cell adhesion molecules, categorised into neural and epithelial varieties. Positive immunostaining for epithelial cadherin was reported within the bronchial epithelium, furthermore, the molecule was localised to epithelial contacts suggesting that it may be present in the intermediate junctions (Montefort *et al.*, 1992). The desmosome and the intermediate junction are the most effective adhesion mechanism between cells.

Tight junctions define the physiological interface between functionally distinct apical and basolateral plasma membrane domains, and more specifically, describe the sealing of adjacent epithelial cells in a narrow band just beneath their apical surface. Tight junctions are formed from macromolecular complexes of proteins and have a continuous distribution around the apical perimeter of epithelial cells. Tight junction proteins such as zonula occludens (ZO)-1, ZO-2, ZO-3, cingulin, symplekin and AF-6 are localized to the cytoplasmic face of the junction and do not participate directly in the formation of an intercellular seal at tight junctions (Anderson *et al.*, 1995; Schneeberger *et al.*, 1992). However, as some of these proteins are anchored to the cytoskeleton, they may be able to regulate tight junction function indirectly. In contrast to the proteins mentioned, occludins and claudins are membrane-spanning proteins that are considered to seal tight junctions by creating homotypic interactions with neighbouring cells (Furuse *et al.*, 1993; Wong *et al.*, 1997). Tight junctions provide two types of barrier role. Firstly, they act by blocking the intercellular space and preventing the movement of material from the lumen of the airway to the serosal area. And secondly, they form a continuous barrier in the external region of the cell membrane separating the molecules on the apical surface from those on the basal lateral surface (Rennard *et al.*, 1991). The

formation of tight junctions by the epithelial cells is of great consequence since it decreases permeability and allows ion transport mechanisms to function efficiently. The high degree of order in the structure of the tight junction, and its ability to undergo rapid structural and functional changes in response to environmental stimuli, has created a great deal of interest in the underlying structural and organisational components of this array.

In addition to these junctional complexes, a number of integrins have been identified in the bronchial epithelium and appear to exert an important role, not only in adhesion but also in epithelial repair following injury. Integrins represent a large family of heterodimeric transmembrane glycoproteins consisting of α- and β-chains. At least seven different integrins are expressed on airway epithelial cells of healthy adults, namely: α2β1, α3β1, α6β4, α9β1, αvβ5, αvβ6, and αvβ8. Studies using mice that express null mutations of these integrins have identified roles for epithelial integrins (Sheppard, 2002). *Table 1.1* displays a summary of the integrins expressed, their known ligands and their distribution within the bronchial epithelium.

Of the integrins listed, α3β1 and α6β4 are receptors for laminins 5, 10 & 11 and are therefore, the only ones to recognise matrix protein ligands, which are present in the normal epithelial basement membrane. However, the pattern of distribution of these integrins is distinct. Expression of α6β4 is completely restricted to the basal surface of basal cells where it forms a fundamental component of hemidesmosomes and is therefore essential for the maintenance of epithelial integrity. Whereas, α3β1 is expressed at the basal surface but is also distributed in lower levels throughout the bronchial epithelium and is directly involved in the organisation of the basement membrane into an ordered structure. Even though α2β1 is primarily associated with collagen I, it is possible that this receptor may interact with collagen IV within the basement membrane. α2β1 and α3β1 are assumed to play important roles in homotypic cell-cell adhesion in the bronchial epithelium, however, this fact is not fully certain (Sheppard, 2003).

Integrin	Known ligand(s)	Distribution
α2β1	Collagen I (IV), tenascin C, echovirus	Ubiquitous with greatest expression on basal cells
α3β1	Laminins 5, 10 & 11	Ubiquitous with highest degree of expression on basal surface of basal cells
α6β4	Laminins 5, 10 & 11	Expression is restricted to basal surface of basal cells
α9β1	Tenascin C, osteopontin, *VCAM-1, L1-CAM, *vWF, FXIII, tissue transglutaminase, fibronectin EIIIA, angiostatin, ADAMS 1,2,3,9 & 15	Ubiquitous with increased expression on basal cells
α5β1	Fibronectin	Ubiquitous but only after injury
αvβ5	Vitronectin, adenovirus, osteopontin	Ubiquitous but mainly expressed on basal cells
αvβ6	*LAP of *1TGF-β1, -3, fibronectin, tenascin C, osteopontin, vitronectin, foot and mouth disease virus	Ubiquitous expression on all cells (dramatically increased by injury)
αvβ8	LAP of TGF-β1, -3, vitronectin	Widespread expression on basal cells

Table 1.1. Expression of integrins on bronchial epithelial cells.
Adapted from review (Sheppard, 2003).

The remaining integrins expressed by the basal cells, including α5β1, α9β1, αvβ5 and α5β6 recognise a wide range of ligands which are not components of the healthy bronchial epithelium. Among these are fibronectin, tenascin C and osteopontin, all of which are highly abundant at the site of epithelial injury. In addition, vitronectin, the principal known ligand for αvβ5, is a plasma protein and is likely to be induced following injury. These integrin receptors play an important role in allowing epithelial cells to rapidly detect and respond to changes in the extracellular matrix that accompany airway inflammation and injury.

Other types of epithelial adhesion molecules include CD44, which represent a family of adhesion molecules that are widely expressed within the bronchial epithelium. CD44 is the principal receptor for the glycosaminoglycan, hyaluronan but may also interact with

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* VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor; LAP, latency-associated peptide; TGF, transforming growth factor.

fibronectin, collagen and some cytokines. It addition to its adhesive role, CD44 is also suggested to play a vital part in epithelial repair as well as cell-cell communication (Knight and Holgate, 2003).

1.1.3. The basement membrane

When considering the integrity of the bronchial epithelium, the role of the basement membrane must not be underestimated. Among other functions, it serves a critical role in the anchorage of the epithelium; the facilitation of adhesion and migration of the epithelial cells; regulation of the phenotype of epithelial cells and it acts as a barrier between the surface epithelium and the underlying mesenchyme (Knight *et al.*, 2003). Under normal conditions, there is little, or no direct contact between epithelial cells and other structural cells, however; there is passive movement of infiltrating inflammatory cells and immune cells between the epithelium and underlying compartments *via* pores in the basement membrane (Howat *et al.*, 2001). The major molecular constituents of basement membranes are collagen and laminin. Collagen IV provides a scaffold for the other structural macromolecules by forming a network *via* interactions between specialized N- and C-terminal domains. Collagen IV and laminin V are predominant constituents of the upper layer of the basement membrane, the lamina densa and the lower lamina reticularis consists of types III and V collagen and fibronectin (Knight and Holgate, 2003). Laminins are heterotrimeric molecules made up by one α-, one β- and one γ- chain. At present, it is recognised that there are five α-chains, three β-chains and three γ-chains. These chains combine into at least 14 different laminins (Amin *et al.*, 2005). The roles of laminins include the mediation of cell attachment as well as the facilitation of cellular proliferation, differentiation and motility (Paulsson, 1992). In the basement membrane, fibronectin exists as an insoluble glycoprotein dimer that plays a role in cell morphogenesis and differentiation through its effects on adhesion, cell shape, and cytoskeletal organisation (Sinkin *et al.*, 1995).

1.1.4. The role of the bronchial epithelium in inflammation

In addition to its role as a protective barrier, the bronchial epithelium is a regulator of airway inflammation. The bronchial epithelium secretes a large array of molecules

including cytokines, chemokines, lipid mediators, growth factors, reactive oxygen species, nitric oxide, matrix proteins and peptide mediators, which demonstrate both pro-inflammatory and anti-inflammatory roles in the airways (Polito *et al.*, 1998). In response to environmental triggers such as endotoxin, cigarette smoke, pollutants, allergens, bacteria and viruses, the epithelium is activated and a large number of mediators are expressed and secreted to a greater extent than normal including: 15-Hydroxyeicosatetraenoic acid (15-HETE), prostaglandin E_2 (PGE_2), fibronectin, endothelin, adhesion molecules, extracellular matrix proteins, eotaxin, chemokines, including IL-8, cytokines and nitric oxide synthase (NOS) (Vignola *et al.*, 1998).

1.1.5. The occurrence of epithelial injury

The bronchial epithelium is considered a prime target for injury due to its exposure to the environment. Inflammation of the epithelium and consequent damage of this delicate lining may be brought about by a number of diverse factors. These include normal exposure to harmful gases, vapours, fumes, aerosols, organic and inorganic particulates, tobacco smoke and diesel exhaust fumes (Mauderley *et al.*, 1987). In addition to pollutants, other damaging factors include proteolytic allergens and microbes, and certain drugs may be responsible for pulmonary toxicity.

1.1.5.1. Damaging effects of the environment

The prevalence of allergic respiratory diseases such as bronchial asthma has increased in recent years, particularly in industrialised countries. The rise in asthma may be explained by changes in environmental factors including indoor and outdoor air pollution. Road traffic with its gaseous and particulate emissions is currently the main contributor to air pollution in most urban areas (Cacciola *et al.*, 2002; Edwards *et al.*, 1994; Wjst *et al.*, 1993). The most abundant air pollutants in urban areas with high levels of vehicle traffic are inhalable particulate matter, nitrogen dioxide and ozone (D'Amato *et al.*, 2005).

Particulate matter remains the most serious air pollution constituent and is consistently associated with adverse respiratory complications. Particulate matter is a mixture of

solid and liquid particles of different size, origin and composition and often contains pollen grains and mould spores. Particle size is critical, since inhalable particles between 5 and 10 μm in aerodynamic diameter are eliminated by mucociliary clearance if the bronchial mucosa is intact; however, particles that are less than 2.5 μm in diameter are able to reach the lower airways and are retained (Churg *et al.*, 1997). It was hypothesised that fine particulate matter found in urban areas, by penetrating deep into the airways is capable of inducing alveolar inflammation which is responsible for variation in blood coagulation and release of mediators involved in acute episodes of respiratory disease (Seaton, 1995). Suggestions were made that transition metals in the particles initiate damage to the airways by the generation of free radicals. In particular, iron appears to be responsible for adverse respiratory problems by the generation of hydroxyl radicals (Smith *et al.*, 1997). Particulate matter derives mainly from diesel exhaust particulate. Although diesel engines emit considerably less carbon dioxide than petrol engines, they emit over 10 times more nitrogen dioxide, aldehydes and inhalable particulate matter (D'Amato *et al.*, 2005). Diesel exhaust particles (DEP) are deposited on the mucosa of the airways and due to their hydrophobic nature, are able to diffuse through cell membranes, bind to a cytosolic receptor complex and modify the growth and differentiation of cells (Diaz-Sanchez, 1997). Studies involving healthy subjects demonstrated an increase in the number of alveolar macrophages, neutrophils and T lymphocytes in BAL following exposure to DEP (Rudell *et al.*, 1996). Following an *in vitro* study investigating the effects of DEP on the interaction of 16HBE 14o$^-$ cells with the cell and matrix microenvironment, Doornaert *et al* (2003) demonstrated that DEP exposure induced: (1) a net dose-dependent decrease in cytoskeleton stiffness through actin fibres, (2) an associated, specific reduction of both α_3- and β_1-integrin subunits extensively expressed on the bronchial epithelial cell surface, (3) a decrease in the level of CD44, which is a major bronchial epithelial cell-cell and cell-matrix adhesion molecule; and (4) an isolated decrease in matrix metalloproteinase (MMP)-1 expression without any change in tissue inhibitor of matrix metalloproteinase (TIMP)-1 or TIMP-2 tissue inhibitors. Given the results of this study, Doornaert *et al* propose that DEPs may facilitate bronchial epithelial cell detachment *in vivo* (Doornaert *et al.*, 2003).

Nitrogen dioxide is abundant in urban and industrialised areas; it is an oxidant pollutant and a precursor for photochemical smog. In conjunction with sunlight and hydrocarbons, nitrogen dioxide is responsible for the production of ozone (D'Amato *et*

al., 2005). Vehicle exhaust emissions are the most significant source of outdoor nitrogen dioxide amongst power plants and sources that burn fossil fuels. Indoors, the most significant exposure to nitrogen dioxide occurs with the use of gas cooking stoves and kerosene heaters. Results of an epidemiological study suggest that exposure to nitrogen dioxide is associated with increased prevalence of asthma and rhinitis and a decrease in lung function in existing asthmatic subjects (de Marco *et al.*, 2002).

Ozone is the main component of photochemical oxidants and 'summer smog' and is predominant in countries of warmer climate, such as those of the meditteranean; however, it is becoming an increasing problem in Europe and indeed in the UK. Inhalation of a high concentration of ozone induces deterioration of pulmonary function and increased hypersensitivity to both specific and non-specific bronchoconstrictor agents and is associated with exacerbation of asthma (Balmes, 1993). Furthermore, it was demonstrated that ozone, in conjunction with nitrogen dioxide modulates airway inflammation by increasing the release of inflammatory mediators from bronchial epithelial cells (Bayram *et al.*, 2001). Ozone also produces an increase in intracellular reactive oxygen species and in epithelial cell permeability, which could in turn lead to penetration of inhaled allergens and toxins in the airways, thus inducing an increase in the release of inflammatory mediators such as IL-1, IL-6, IL-8 and TNF. In addition, ozone has been shown to be detrimental to mucociliary clearance function (Schlesinger *et al.*, 1987). Studies have indicated a significant increase in the levels of inflammatory mediators such as IL-6, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF) and fibronectin in BAL fluid in response to ozone (Balmes *et al.*, 1997). Further studies involving the analysis of BAL fluid have demonstrated an increased abundance of neutrophils, and cytokines and evidence of increased epithelial permeability following short-term exposure to ozone in subjects with mild asthma compared to healthy subjects (Basha *et al.*, 1994; Scannell *et al.*, 1996). Ozone inhalation also reduces exercise tolerance in non-asthmatic athletes (Adams *et al.*, 1983). Repeated daily, short-term exposure of healthy subjects to ozone has been implicated in the deterioration of pulmonary function and inflammatory response (Christian *et al.*, 1998).

Pollen grains are the primary carriers of pollen allergens and are implicated in hayfever, whereby typical symptoms are designated to the eyes, nose and nasopharynx. However, the direct association of pollen with asthma is debatable, since intact pollen grains are

typically more than 10 μm in diameter and therefore do not enter the lower airways as they are eliminated by mucociliary clearance (D'Amato *et al.*, 2002). However, the release of pollen allergens from ruptured pollen grains into the atmosphere could provide a plausible explanation for this, as their small size would allow them to penetrate the peripheral airways following inhalation. Moreover, pollen allergens could combine with other small particles in the atmosphere, such as diesel exhaust particulate, which have the potential to penetrate into the airways and be retained (Knox *et al.*, 1997).

1.1.5.2. Damaging side effects of drugs

Pulmonary drug toxicity is increasingly being diagnosed as a cause of acute and chronic lung disease. Numerous agents including noncytotoxic as well as cytotoxic drugs have the potential to cause pulmonary toxicity (Rossi *et al.*, 2000). Drug-induced pulmonary disease is not a single disorder, but rather, a common clinical problem in which a patient without previous pulmonary disease develops respiratory problems, chest X-ray changes, deterioration of pulmonary function, and/or histological changes while receiving drug therapy. Over 150 drugs have been reported to cause pulmonary disease, many of which are thought to provoke a hypersensitivity response. The most common histopathological processes underlying pulmonary drug toxicity include pneumonitis and fibrosis.

Patients undergoing chemotherapy are at particular risk of developing respiratory complications (Hagarty and Chisholm, 2002). An early inflammatory interstitial pneumonitis may be associated with the use of cytotoxic drugs such as methotrexate, bleomycin, busulphan, mitomycin, procarbazine and carmustine or non-cytotoxic drugs such as amidarone. Late onset pulmonary disease is more common and usually takes the form of pulmonary fibrosis. The lung is a common site of infection in cancer patients and the immunocompromised patient is vulnerable to a whole host of inhaled microorganisms.

Case studies have shown that safe medications such as the cyclooxygenase-2 (COX-2) inhibitors, which are widely prescribed for pain and arthritis, have been implicated in respiratory complications such as dyspnoea, severe pulmonary fibrosis and hypoxemia

(Chia *et al.*, 2003). With respect to the prostaglandin pathway, inhibition of COX-2 by various drugs such as celecoxib or ibuprofen results in inhibition of PGE_2 production. A decrease in PGE_2 is associated with increased fibroblast proliferation, which contributes to the propensity for pulmonary fibrosis (Bonner *et al.*, 2002).

1.1.5.3. Direct epithelial damage by house dust mite proteases

High or persistent exposure to potential allergens, particularly the house dust mite antigen presents a major risk factor for the development of allergic airway diseases (Platts-Mills *et al.*, 1992). The Pyroglyphidae family of the common house dust mite is associated with human atopic disease. In Europe, the predominating species of pyroglyphidae are *Dermatophagoides pteronissinus* (Der p) (Roche *et al.*, 1997). Allergens from these mites are secreted in their faeces (Tonnel *et al.*, 1983). House dust mites are ubiquitous and their growth is greatly influenced by relative humidity and temperature. Food sources of mites include skin scales or fungi that grow on them.

Proteases produced by house dust mites are able to decrease the barrier function of the epithelial cell layer. One of the major house dust mite allergens, Der p1 has been shown to have cysteine protease activity (Chua *et al.*, 1988; Simpson *et al.*, 1989) and has been associated with epithelial cell desquamation, facilitation of transport of allergens across cultured epithelial cell layers and release of cytokines (King *et al.*, 1998; Robinson *et al.*, 1997; Wan *et al.*, 2000; Winton *et al.*, 1998). The proteases may disrupt the tight junctions between epithelial cells and lead to the complete desquamation of the epithelial cell layer, hence facilitating the passage of allergens across the epithelial surface (Wan *et al.*, 1999). In particular, the occludin and claudin families of tight junction adhesion proteins were recognised as targets. Epithelial desquamation and a decline in epithelial integrity as a result of protease activity by house dust mite allergens leads to further exposure and susceptibility to allergens. Furthermore, it allows allergens without proteolytic activity to gain access to the submucosa. Studies demonstrated that Der P1 activity was partially inhibited by cysteine protease and serine protease inhibitors that are naturally produced by the normal human lung, indicating that epithelial cells from asthmatic subjects have inherently lower levels of antiproteases to neutralise the proteolytic activity of house dust mite allergens (Mattoli, 2001).

In response to proteases present in house dust mite extracts, bronchial epithelial cells have been shown to increase the release of proinflammatory cytokines such as IL-6 and GM-CSF, the chemokines IL-8 and eotaxin and the platelet derived growth factor (PDGF) (Asokananthan *et al.*, 2002; Borger *et al.*, 1999; Kauffman *et al.*, 2000); the release of which, are mediated by protease activated receptors (PARs) that are expressed on bronchial epithelial cells (Cocks *et al.*, 1999; D'Andrea *et al.*, 1998).

There is some evidence that Der P1 can trigger sensitisation to the allergen and then repeated exposure can lead to an allergic response in sensitised individuals (Sharma *et al.*, 2003). One hypothesis is that asthma in house dust mite sensitive patients may be caused by recurrent inhalation of live dust mites which reside in the bronchioles for long periods of time and in order to provide themselves with a food source, they excrete proteolytic enzymes, including Der p1 which promote epithelial shedding by releasing cells from the basement membrane, allowing the mites to feed on the shed epithelial cells. Consequent removal of the intact bronchial epithelium exposes the underlying mesenchyme to dust mite proteins and other allergens, triggering sensitisation to these proteins. Eventually, repeated infestation provokes an allergic response which manifests itself as asthma attacks (van Woerden, 2004).

1.1.6. Mechanisms of normal bronchial epithelial repair

Early techniques to denude the airways *in vivo* in order to study normal epithelial repair involved neck surgery. This proved to be difficult and traumatic and resulted in damage to the basement membrane as well as local bleeding (Dunnill, 1960; Wilhelm, 1953). Thereafter, many studies relied upon bronchial epithelial cell culture (Zahm *et al.*, 1991). However, a number of limitations were established with *in vitro* culture techniques, including the inability of the cells to differentiate (particularly in submerged cultures) and the variability that occurs with cell source and culture conditions. Moreover, it was evident that vital components from the airway microcirculation are lost in the *in vitro* model. Progress was therefore made with the development of a novel technique to create well-defined epithelial denudation *in vivo*, without causing bleeding or disruption to the basement membrane. This was carried out in the guinea pig, whereby an oral catheter was introduced into the tracheal lumen, thus, avoiding surgery or any disturbance to the tracheal mucosa, except for the required gentle denudation

effect (Erjefalt *et al.*, 1993; Erjefalt *et al.*, 1991). Mechanical deepithelialisation using an orotracheal steel probe created a wound of 800 μm wide. Immediately after epithelial removal, secretory, ciliated and presumably basal epithelial cells at the border of the denuded zone were shown to dedifferentiate, flatten and migrate rapidly to cover the denuded basement membrane (*figure 1.2*). The ciliated cells were shown to dedifferentiate into flat, poorly differentiated epithelial cells that migrate. These cells prove to be extremely adaptive since the cilia become internalised as a result of the differentiation into flattened cells. During the first few minutes after denudation, the speed of migration was determined to be several μm per minute, thus, acute repair is exceptionally quick compared to that observed in the *in vitro* model (Erjefalt *et al.*, 1997). After 15 minutes, cells started to migrate over the denuded area, and after 8 hours most of the denuded area was covered by a flat undifferentiated epithelium (Erjefalt *et al.*, 1995). This implies an important role for specific *in vivo* factors in the repair process. It was demonstrated that, almost immediately after removal of the epithelium, there was evidence of extravasation and bulk entry of plasma from the microcirculation beneath the denuded, but intact basement membrane (Erjefalt *et al.*, 1994; Persson *et al.*, 1998).

It was recognised that plasma exudation created a fibronectin and fibrin rich gel to cover the denuded basement membrane and support the migration of the epithelial cells. There was also an abundance of leukocytes within the gel suggesting an important role for these cells in the rapid repair of the epithelium. It is thought that there are several sources for these cells including, the epithelium itself, the tissue underlying the basement membrane and the microcirculation. Another important observation was that eosinophils were found to be present in the denuded area. Eosinophils are capable of producing several factors including TGF-β, PDGF, hepatocyte growth factor (HGF) and heparin-binding growth factor (HB-EGF), all of which may promote wound healing. The repair process in the guinea pig proved to be extremely rapid with reepithelialisation occurring within 8-15 hours. Proliferation of cells in order to repopulate the wounded area occurred at approximately 15 hours when cell migration was complete. Remarkably, at 30 hours, the new epithelium consisted of 2-3 layers; and 5 days following denudation, a normal, ciliated epithelium was established (Erjefalt *et al.*, 1994).

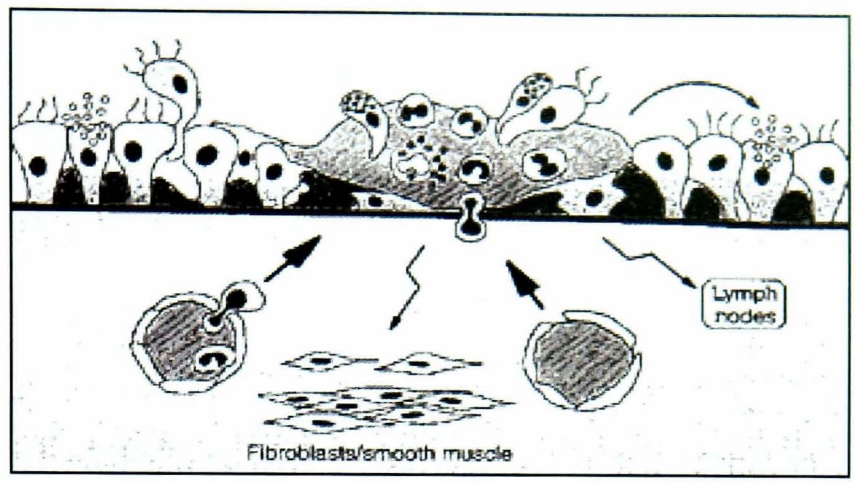

Figure 1.2. Bronchial epithelial repair. Immediately after injury, columnar cells (lightly shaded) and basal cells (darkly shaded) are involved in a continuous resealing of the denuded basement membrane. The repair process is aided by plasma exudation (thick arrows) (Erjefalt *et al.*, 1997).

In addition to mechanical wounding *in vivo*, a second model was employed to investigate epithelial repair. This involved the induction of epithelial damage by allergen-challenge. Guinea pigs were subjected to non-traumatic, intratracheal mucosal super-fusion of ovalbumin, prior to removal of the trachea and examination of tissue (Erjefalt *et al.*, 1997). A marked increase in neutrophils was demonstrated in tracheal lavage fluid and in tissue and was strongly associated with the challenge-induced areas of epithelial damage and restitution; eosinophils were elevated in the tracheal lumen whereas the surrounding tissues displayed a reversed pattern; gels rich in fibrinogen, neutrophils and eosinophils were present in areas of epithelial damage; clusters of free eosinophil granules, termed Cfegs, released through lysis of eosinophils; and neutrophils displaying long cytoplasmic protrusions were abundant in areas of epithelial damage. Taken together, these findings demonstrate the association of inflammation with epithelial damage-restitution processes in allergic airways.

1.1.7. The role of fibrin in bronchial epithelial repair

In response to extravascular injury, the extrinsic coagulation cascade is activated (Davie *et al.*, 1991). In brief, the cascade is initiated by the activity of tissue factor (TF). TF is found at extravascular sites only and acts as a receptor for factor VII (FVII), which is released from damaged vessels and is able to bind to TF to induce coagulation. TF binds and activates FVII, which in turn, activates factor X (FX). Activated factor X (FXa) activates prothrombin to generate thrombin, which cleaves soluble fibrinogen to

insoluble fibrin with subsequent removal of fibrinopeptide A (FPA) and fibrinopeptide B (FPB). Finally, the fibrin clot is stabilised by covalent cross-linking by the plasma transglutaminase factor XIII (FXIII). The resultant fibrin clot provides a provisional matrix for cell migration (*figure 1.3*).

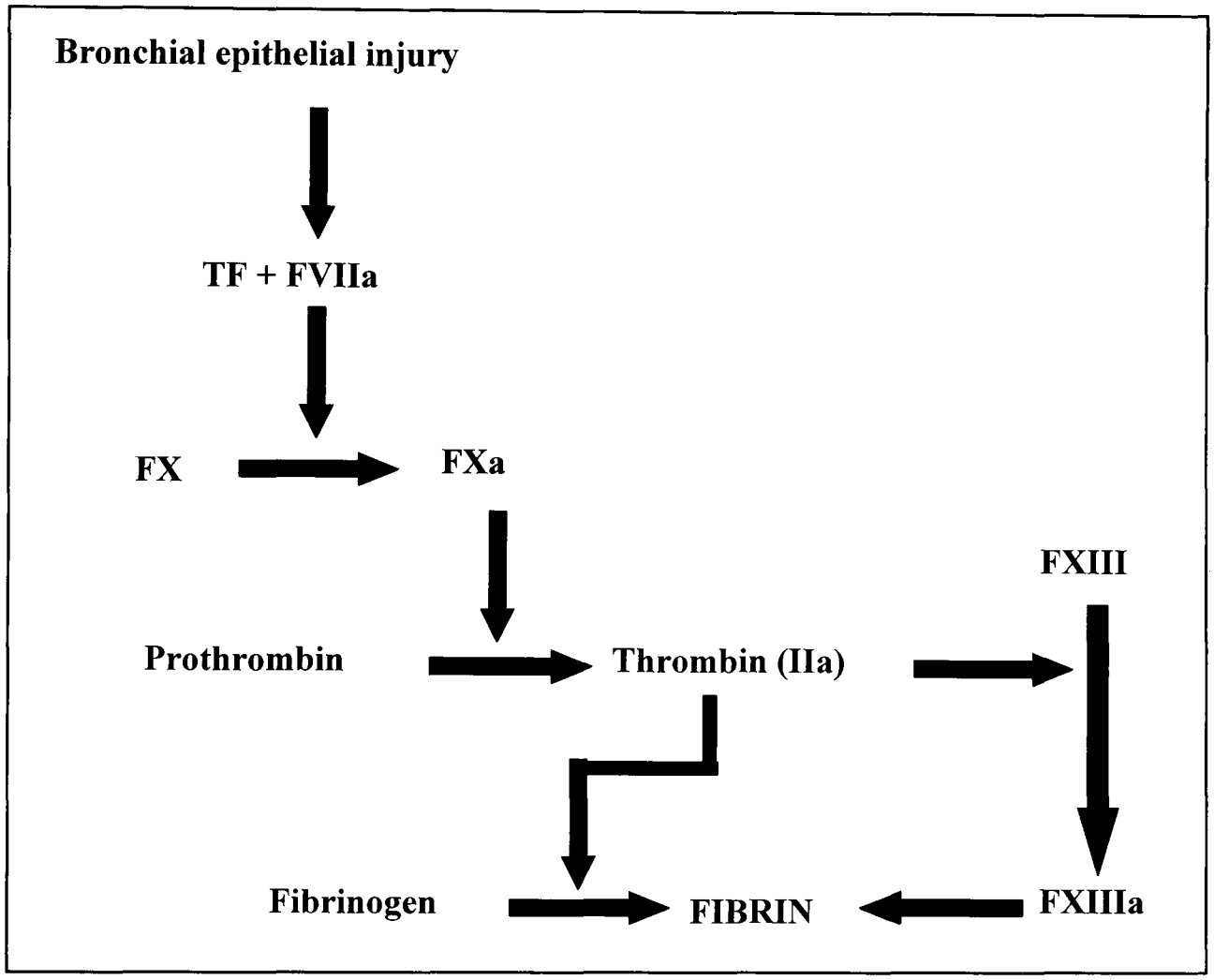

Figure 1.3. A simplified schematic representation of the extrinsic coagulation cascade.

A fibrin provisional matrix is required for the successful repair of epithelial cells as it provides a surface onto which cells are able to migrate, proliferate and restore tissue integrity (Laplante *et al.*, 2001). The importance of fibrin is also demonstrated by the use of fibrin-containing sealants in severe wounds to provide a matrix to promote skin repair (Singer *et al.*, 1999). A fibrin provisional matrix forms when damage to the blood vessel or to the tissue around it occurs. Evidence for a fibrin matrix in the airways came from a study of bronchial biopsies comparing asthmatic and normal subjects. Where the epithelium was denuded in the symptomatic group of patients a fibrin-like material associated with platelets was found where there was no epithelial coverage (Jeffery *et al.*, 1989). When the epithelium becomes damaged the neighbouring cells spread by reorganisation of the actin cytoskeleton and the extension of lamellaepodia.

When epithelial cells migrate in response to damage they move in sheets. The migration is controlled by activation of extracellular signal regulated kinase (ERK) mitogen-activated protein kinase (MAPK) (Matsubayashi *et al.*, 2004). The change in structure of the cells allows the migration into the denuded area and once migration has occurred, the cells then proliferate (Zahm JM et al, 1991). *In vivo* studies demonstrated that mechanical removal of the epithelial lining in the guinea pig resulted in rapid induction of plasma exudation from the underlying microcirculation followed by rapid production (within 30 minutes) of a fibrin-rich gel to cover the denuded basement membrane, displaying fibronectin-like immunoreactivity (Erjefalt *et al.*, 1993). In addition to providing a matrix to support migration of the epithelial cells, the fibrin clot also provides a matrix scaffold for the recruitment of a host of infiltrating leukocytes, adhesive plasma-derived proteins, fibronectin, eosinophils and growth factors, all of which are believed to promote wound healing. Thus, the role of fibrin in epithelial repair appears to be invaluable. However, net fibrin formation is dependent on fibrinolysis, the breakdown of the fibrin matrix in addition to fibrinogenesis, the formation of a fibrin matrix and it is imperative that the fibrin clot is broken down just as efficiently as it was deposited (Clark, 2001). Therefore, once fibrin has performed its duty, it must be degraded to soluble fragments as inadequate removal may lead to fibrosis. Fibrinolysis occurs in a sequence of distinct steps. Firstly, plasminogen is cleaved by plasminogen activators to generate plasmin. This event is primarily mediated by tissue-type plasminogen activator (t-PA). Plasmin has the capacity to reorganise and disintegrate the fibrin clot, until the final step of dissolution. The D-dimer molecule derives from cross-linked fibrin only and is therefore an indication of both fibrin formation and its dissolution.

1.1.8. The role of integrins in wound repair

Integrins have been shown to play an important role in the spreading, migration and proliferation of epithelial cells (Damjanovich *et al.*, 1992; Montefort *et al.*, 1993). Furthermore, integrins mediate adhesion of epithelial cells to the extracellular matrix (Bosman, 1993; Quaranta, 1990). Epithelial cells are known to express integrins for collagen, laminin-1 and fibronectin found in basement membranes (Pilewski *et al.*, 1997). Moreover, one report demonstrates that one variant of laminin, laminin-2, is

expressed in the basement membrane of the airways in subjects with chronic asthma but not in non-asthmatic subjects (Altraja *et al.*, 1996). It has been suggested that integrin receptors found on bronchial epithelial cells, such as $\alpha_2\beta_1$ and $\alpha_6\beta_1$ bind laminin-2 less well than laminin-1 (Brown *et al.*, 1994). Thus, it is possible that expression of matrix proteins in asthmatic airways decreases epithelial cell migration and repair over the basement membrane.

The majority of studies involving the participation of integrins in wound repair have been confined to squamous epithelia, such as the skin. Cutaneous wounds display a provisional matrix that is rich in integrin ligands including fibronectin, osteopontin and tenascin (Sheppard, 2003). In response to epithelial injury there is a major change in the distribution and expression of epithelial integrins (*Figure 1.4*). Expression of $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha6\beta4$, $\alpha v\beta5$, and $\alpha v\beta6$ is significantly upregulated at the wound edge suggesting an important role for these integrins in the regulation of normal wound repair.

White *et al* (1999) investigated the role of integrins in an *in vitro* model of wound repair. Wounds were created in monolayers of 16HBE 14o$^-$ cells cultured on either collagen IV, laminin-1 or laminin-2 matrix. Wound repair was inhibited by a monoclonal antibody to β_1-integrin, furthermore, treatment with a monoclonal antibody to α_2-, α_3- and α_6-integrin blocked wound repair in monolayers of cells grown on collagen-IV but not laminin-1 or laminin-2. These data demonstrate that β_1-integrin subunit function is required to permit migration and spreading of epithelial cells, and that α-integrin subunits alone do not mediate migration of epithelial cells grown on either laminin-1 or laminin-2. These differences may become important if the matrix protein composition of airway basement membrane is altered in disease states such as asthma (White *et al.*, 1999).

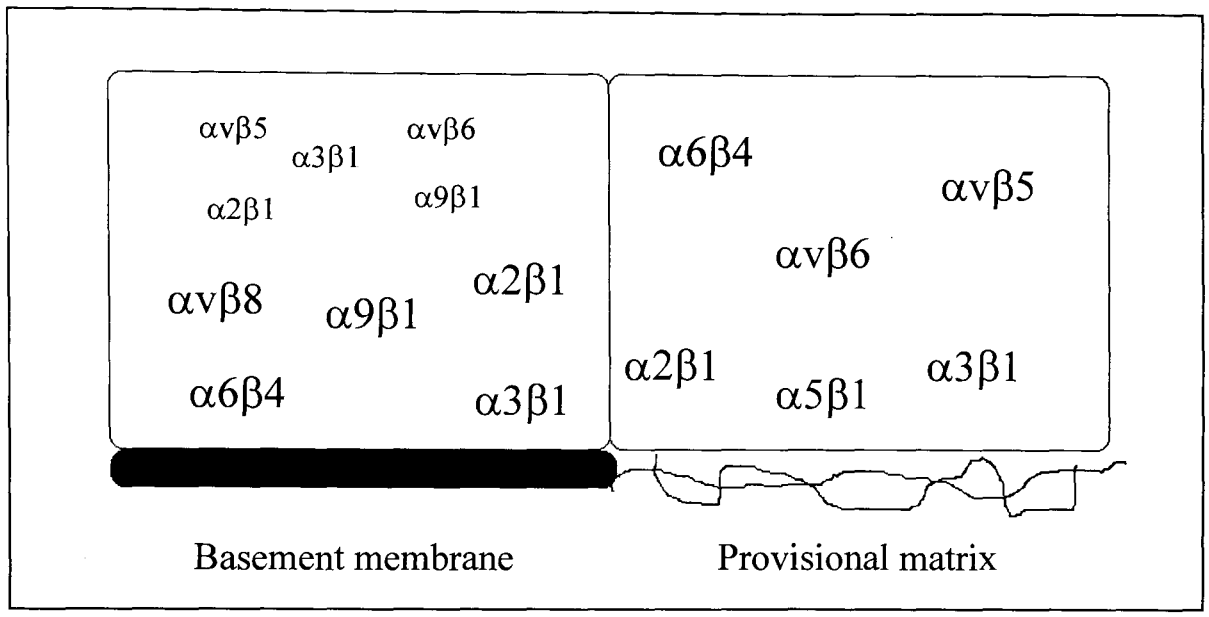

Figure 1.4. Changes in the level and distribution of expression of integrins in the airway epithelium in response to injury. Normal, unwounded epithelium is shown on the left, and the right hand side represents a theoretical site of denudation. Levels of expression are depicted by the size of the text characters corresponding to individual integrins (Sheppard, 2003).

1.1.9. The role of trefoil factor family peptides in bronchial epithelial repair

Three members of the trefoil factor family peptides (TFF-peptides), known as TFF1, TFF2 and TFF3 are found in humans (Hoffmann *et al.*, 1993). These peptides are secreted from the lumen of a variety of mucous epithelia. In combination with mucins, these peptides are an important constituent of mucous gel. TFF3 is the predominant TFF-peptide within the bronchial epithelium. It is primarily released from the submucosal gland and to a lesser extent from the goblet cells. It is well established that all three TFF-peptides act as motogens during *in vitro* wound healing assays in various gastrointestinal cell lines (Dignass *et al.*, 1994; Marchbank *et al.*, 1998; Wilson *et al.*, 1997). Using an *in vitro* model of wound repair involving the culture of a cell line of human bronchial epithelial cells, Oertel *et al* (2001) discovered that all three TFF-peptides act as motogens, stimulating rapid repair of the wounded epithelium, and in the presence of epidermal growth factor (EGF), this effect is synergistic (Oertel *et al.*, 2001).

In order to identify a role for TFF3 in differentiation of the human airway surface epithelium, Lesimple *et al.* (2006) analysed the temporal expression pattern of TFF3, goblet cells and ciliated cell markers during human airway epithelial regeneration using

in vivo humanised xenograft and *in vitro* air-liquid interface (ALI) culture models. They observed that TFF3, goblet cells and ciliated cell markers were expressed in a well-differentiated airway epithelium. The addition of exogenous recombinant human TFF3 to epithelial cell cultures prior to the initiation of differentiation resulted in no change in goblet cells but induced an increase in the number of FOXJ1 positive cells, indicating ciliagenesis and an increase in the number of ciliated cells. Furthermore, the effect on ciliated cell differentiation was reversed by specific epidermal growth factor receptor (EGFR) inhibition indicating that TFF3 is able to induce ciliagenesis and to promote airway epithelial ciliated cell differentiation *via* an EGFR-dependent pathway (Lesimple *et al.*, 2006).

1.1.10. The role of nitric oxide in bronchial epithelial repair

Nitric oxide (NO), is a gaseous molecule generated endogenously during the conversion of the amino acid L-arginine to L-citrulline by a family of NO synthases (NOSs) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Constitutive and inducible forms of NOS, abbreviated to cNOS and iNOS respectively, have been described and are both present in the airways with divergent effects. iNOS expression is induced by proinflammatory cytokines and releases large quantities of proinflammatory NO in a sustained manner (Morris *et al.*, 1994). However, calcium-dependent cNOS activity, represented by neuronal (nNOS) and endothelial (eNOS) isoforms respectively, leads to the rapid release of small amounts of bronchoprotective NO (Folkerts *et al.*, 1998).

The bronchial epithelium continuously produces NO, largely due to constitutive expression of iNOS (Xu *et al.*, 2006). Although iNOS is typically induced under conditions of infection or inflammation, constitutive iNOS expression within the airway may serve functions in innate host defence (Xu *et al.*, 2006), or regulation of epithelial ion transport (Duszyk, 2001; Hardiman *et al.*, 2004). Gookin *et al* (2001) investigated the effects of iNOS on porcine ileum in an *in vitro* study of epithelial repair. They demonstrated that iNOS is constitutively expressed by the ileal epithelium and mediates a significant increase in endogenous NO synthesis following injury suggesting that NO is a key mediator of epithelial repair (Gookin *et al.*, 2002). Similarly, in an *in vivo* model of cutaneous wound repair in mice, Yamasaki *et al* (1998) demonstrated that iNOS is expressed in the healing wound and that inducible production of nitric oxide

accelerates the rate of excisional wound repair (Yamasaki *et al.*, 1998). The protective effect of NO in wound repair may be due to the ability of NO to promote cell migration in various cell types (Jadeski *et al.*, 2003; Kawasaki *et al.*, 2003), however, little is known about the importance of iNOS in bronchial epithelial repair. Bove *et al* (2007) investigated the involvement of epithelial iNOS in bronchial epithelial cell migration and repair in an *in vitro* wound model using an exogenous NO donor and stable transfection with iNOS in an attempt to mimic continuous NO production by bronchial epithelial cells *in vivo*. They demonstrated that low, physiologically relevant concentrations of NO promote bronchial epithelial cell migration and wound repair, involving cGMP-dependent expression and activation of MMP-9 (Bove *et al.*, 2007).

1.1.11. The role of relaxin in bronchial epithelial repair

Relaxin, a polypeptide of 6 kDa, belongs to the insulin-like growth factor family and was first described in 1926 by Frederick L. Hisaw (Hisaw, 1926). Relaxin was originally identified as a pregnancy related hormone and since then, the polypeptide has been attributed to a variety of tissues, including the lung (Bani, 1997; Cheah *et al.*, 1980). Bani *et al* (1997) demonstrated in an ovalbumin-sensitised guinea pig model of asthma, that relaxin has the potential to reduce the severity of respiratory abnormalities. This was signified by histological alterations, mast cell degranulation and leukocyte infiltration, suggesting an anti-asthmatic property of relaxin (Bani *et al.*, 1997). Results of a clinical study revealed that decreased asthma severity correlates with elevated levels of relaxin in pregnant women (White *et al.*, 1989). In addition to its involvement in the inflammatory process in asthma, relaxin has also been shown to stimulate epithelial proliferation in rat cervix (Burger *et al.*, 1998). Furthermore, relaxin was shown to inhibit the proliferation of human lung fibroblasts and to reduce fibrosis in the murine lung model (Unemori *et al.*, 1996). Thus with a combination of enhancing proliferation of epithelial cells and inhibiting fibroblast proliferation, relaxin may play a vital role in the repair of the injured lung. In addition to these beneficiary effects of inhibiting fibrotic events and stimulating epithelial cell growth, relaxin was reported to promote effective mucociliary clearance by increasing ciliary beating frequency (CBF). This effect was mediated via regulation of cAMP, and protein kinase A (PKA) (Sanderson *et al.*, 1989). Wyatt *et al* (2002) investigated the effects of relaxin on bronchial epithelial cell function using an *in vitro* model of wound repair and ciliary

beating. They demonstrated that relaxin accelerated the rate of wound repair compared to control and caused a significant stimulation of CBF in bronchial epithelial cells and speculated that relaxin promotes epithelial repair by increasing migration and CBF *via* PKA-dependent mechanisms (Wyatt *et al.*, 2002).

1.1.12. The role of glycoproteins in bronchial epithelial repair

Given that many adhesion molecules and cell surface receptors for growth factors and cytokines are glycoproteins, and the fact that epithelial damage results from a loss in epithelial integrity, recent interest has been taken in the role of glycoproteins in wound repair. Several studies support the theory that oligosaccharide moieties are crucial for the function of some of these glycoproteins and that variation in their glycosylation pattern often leads to changes in their function (Feige *et al.*, 1988; Leconte *et al.*, 1992; Rands *et al.*, 1990). Oligosaccharides on cell surface receptors have roles in cell adhesion, migration (Schnaar, 1991) and proliferation (Chammas *et al.*, 1994). Xiantang *et al* (2000) demonstrated in the guinea pig model that glycosylation profiles in the bronchial epithelium change over time following mechanical wounding (Xiantang *et al.*, 2000). Kauffmann *et al* (1996) reported an under-representation of carbohydrate structure with terminal fucose in asthmatic subjects, with a correlation between this deficiency and the severity of the disease. These data suggest that defects in epithelial repair in asthmatic subjects may be due, in part, to improper glycosylation of bronchial epithelial cells (Kauffmann *et al.*, 1996). Allahverdian *et al.* (2006) investigated the role of sialyl Lewis^x in bronchial epithelial repair. Sialyl Lewis^x is a fucose-containing tetrasaccharide present on membrane-bound and secreted proteins, is a ligand for E-cadherin, which is found in tight junctions and thus mediates cell-cell interaction (Lowe *et al.*, 1990; Walz *et al.*, 1990). Using immunohistochemistry, an increased expression of sialyl Lewis^x was demonstrated in areas of damaged bronchial epithelium compared with the intact regions. Reverse transcriptase polymerase chain reaction (RT-PCR) revealed an increase in expression of sialyl Lewis^x with time *post*-wounding. Sialyl Lewis^x accelerated wound repair and this effect was completely reversed by the use of an inhibitory antibody to the carbohydrate, suggesting an essential role for sialyl Lewis^x in bronchial epithelial repair (Allahverdian *et al.*, 2006). Prior to this, similar *in vitro* studies revealed that the binding of specific lectins to their carbohydrate residues impaired the normal wound repair response in a bronchial epithelial cell model (Adam

et al., 2003). These results were in agreement with a study by White *et al* (2001), whereby, the addition of the lectin from *triticum vulgaris* to repairing cultures of a bronchial epithelial cell line resulted in inhibition of wound repair (White *et al.*, 2001). Lectins are naturally occurring glycoconjugate-binding molecules that can be isolated from a wide variety of plants and animals and provide selective tools to identify or inhibit specific glycoconjugate sites; for example, the lectin from the bacterium *Pseudomonas aeruginosa* has been shown to bind to fucose residues on the bronchial epithelium, resulting in the cessation of ciliary beating (Adam *et al.*, 1997). It is suggested that binding through this lectin could be a mechanism of attachment for the bacteria, which can then proliferate. The lectin from *Triticum vulgaris* has also been shown to bind to the apical surfaces of bronchial ciliated cells and inhibit ciliary beating (Adam *et al.*, 1995). It must be noted that although this lectin would not normally be encountered in the airway, it demonstrates the importance of blocking carbohydrate residues on physiological processes such as epithelial repair. Li *et al.* (2000) have demonstrated that increased lectin binding is detected in guinea pig tracheal epithelial cells following damage, denoting an altered glycoconjugate profile as a result of damage, subsequent repair, or both (Li *et al.*, 2000). Collectively, these studies provide strong evidence that carbohydrates are involved in epithelial repair of the damaged epithelium *via* surface interactions and that the use of lectins provide a good method of probing carbohydrate moieties on the surfaces of cells to establish their role in fundamental processes such as bronchial epithelial repair and in the repetitive cycles of injury and repair seen in asthma.

1.2. Asthma

Asthma is a respiratory disease characterised by recurrent respiratory symptoms, reversible variable airway obstruction, airway inflammation and increased airway responsiveness (Holloway *et al.*, 1999). T-helper 2 (Th2)-mediated inflammation is considered to provide the basis for altered structure and function that leads to airway hyperresponsiveness (AHR) and variable airflow obstruction (Davies *et al.*, 2002). Allergy or 'atopy' describes a disorder involving immunoglobulin E (IgE) antibody responses to ubiquitous allergens and is commonly associated with asthma. Although atopy is considered an important risk factor for asthma, a recent birth cohort study has shown that induction of specific IgE responses and development of childhood asthma

are determined by independent factors (Lau *et al.*, 2000). Even though asthma has a strong genetic basis, the reason behind the inherent fragility of the asthmatic epithelium is not known. The increase in prevalence of this disease over that last 30 years has occurred in too short a time for new genetic changes to be responsible. Asthma may therefore be considered a complex disease in which the interaction between both genetic and environmental factors plays a fundamental role both in the pathogenesis and in the development of the disease. It has been hypothesised that atopy/airway inflammation and altered structure and function of the bronchial epithelium are parallel but interacting factors (Davies *et al.*, 2002; Djukanovic, 2000). For asthma to develop as a chronic disease, genetic and environmental factors that drive each of these components are required. Fundamental to this is the concept of aberrant signalling between the bronchial epithelium and the underlying mesenchyme and persistent activation of the epithelial mesenchymal trophic unit (EMTU).

1.2.1. Inflammation in asthma

Inflammation is classified by four cardinal signs: *calor* and *rubor* (heat and redness, due to vasodilatation), *tumour* (swelling, due to plasma exudation and oedema) and *dolor* (pain, due to sensitisation and activation of sensory nerves). It is now recognised that inflammation is also characterised by infiltration with inflammatory cells and that these will differ, depending on the inflammatory process (Barnes, 2003). Inflammation is an important defence response that protects the body against invasion from microorganisms and the effects of harmful, external toxins. The inflammation in allergic asthma is characterised by the fact that it is driven by exposure to allergens through IgE-dependent mechanisms, resulting in infiltration with eosinophils. The inflammatory process provides an acute defence against infection. However, in allergic asthma, the inflammatory response is activated inappropriately by exposure to allergens, such as house dust mite and pollen proteins that induce eosinophilic inflammation that is harmful rather than beneficial. Normally, such an inflammatory response would eliminate the invading parasite, but in allergic disease, the invading stimulus persists and the normally acute inflammatory response becomes converted into a chronic inflammation, which may have structural consequences in the bronchial wall (*section 1.2.4*). The fact that there is a defect in the normal reparative mechanisms of the

bronchial epithelium signifies that it remains in an active state and additional proinflammatory mediators are released (Holgate *et al.*, 1999).

In severe asthma cases, resulting in death from acute asthma attacks, the airway lumen is occluded by a mucous plug composed of plasma proteins exuded from airway vessels and mucous glycoproteins secreted from surface epithelial cells (Barnes, 2003). The airway wall is oedematous and infiltrated with inflammatory cells, including eosinophils and lymphocytes. Severe asthma differs to normal allergic asthma in that the inflammatory profile is commonly associated with greater involvement of neutrophils (Holgate *et al.*, 2006).

The airway epithelium is invariably shed in a patchy manner and clumps of epithelial cells may be found in the airway lumen. Occasionally, there have been opportunities to examine the airway of asthmatic patients who die accidentally and similar, although less marked changes have been identified (Dunnill, 1960). More recently, it has been possible to examine the airways of asthmatic patients by fibre-optic and rigid bronchoscopy, bronchial biopsy and BAL. Direct bronchoscopy reveals that the airways of asthmatics are often reddened and swollen, indicating acute inflammation. BAL analysis has revealed an increase in the numbers of lymphocytes, mast cells and eosinophils and evidence for activation of macrophages in comparison with non-asthmatic controls. Similarly, biopsies have revealed evidence for increased numbers and activation of mast cells, macrophages, eosinophils and T-lymphocytes (Bousquet *et al.*, 2000; Busse *et al.*, 2001). These changes are found even in cases of mild asthma, suggesting that asthma is an inflammatory condition of the airways. And the presence of neutrophils in severe asthma and exacerbations of asthma is associated with viral and bacterial infection.

1.2.2. Inflammatory mediators

Many different mediators have been implicated in asthma and they may exert a variety of effects on the airways, which could account for all of the pathological features of asthma (*table 1.2*). Because each mediator has many effects, the role of individual mediators in the pathophysiology of asthma is not yet clear (Barnes *et al.*, 1998).

Source of inflammatory mediator	Inflammatory mediator	Inflammatory effects
Inflammatory cells Mast cells Eosinophils Th2 cells Basophils Platelets Neutrophils ***Structural cells*** Epithelial cells Endothelial cells Smooth muscle cells Fibroblasts Nerve cells	***Lipid mediators*** LTC_4 LTD_4 LTE_4 PAF Prostaglandins	Bronchoconstriction Increased AHR Recruitment of inflammatory cells (e.g. eosinophils)
	Proinflammatory cytokines IL-1β IL-4 IL-5 IL-6 IL-9 IL-13 TNF-α GM-CSF Lymphokines	Recruitment, differentiation and survival of eosinophils Survival of mast cells Activation of β-lymphocytes to produce IgE Amplification of the inflammatory response Increased AHR Activation of proinflammatory transcription factors (e.g. NF-κB and AP-1)
	Regulatory cytokines IL-10 IL-12 IL-18 IFN-γ	Inhibition of the inflammatory response
	Chemokines **Eotaxin** Eotaxin-2 **Eotaxin-3** RANTES MCP-1 MCP-3 MCP-4 MDC TARC	Recruitment of eosinophils Recruitment of Th2 cells Increased AHR

Table 1.2. A summary of the key cells and inflammatory mediators that are involved in asthma and their effects in the airway. LT, leukotriene; PAF, platelet activating factor; NF-κB, nuclear factor-κB; AP-1, activator protein-1; IFN, interferon; MCP, monocyte chemoattractant protein; MDC, monocyte derived chemokine; TARC, thymus and activation regulated chemokine.

1.2.3. The consequence of epithelial damage in asthma

In asthma, the bronchial epithelium has increased susceptibility to injury and normal repair processes are compromised (Adam *et al.*, 2003). Epithelial shedding is considered a major characteristic feature and pathogenic mechanism in asthma (Erjefalt *et al.*, 1997). The asthmatic epithelium has been shown to display a loss of columnar epithelial cells, which leads to a loss of epithelial integrity (Montefort *et al.*, 1992). Defective desmosome structure and function has been proposed to lead to a suprabasal plane of cleavage. The occurrence of shedding is supported by findings of elevated numbers of epithelial cells in sputum and BAL fluid. Studies performed by Naylor in the 1960's demonstrated the presence of increased numbers of epithelial cell clusters known as 'creola bodies' in the BAL fluids from asthmatics representing significant shedding of columnar epithelial cells. Furthermore their numbers were further increased during exacerbations of the disease (Naylor, 1962). Evidence for epithelial shedding has also been supported by histological observations in bronchial biopsies and autopsies (Dunnill, 1960; Laitinen *et al.*, 1985; Ollerenshaw *et al.*, 1992). Epithelial shedding results in a poorly differentiated 'activated' epithelium, consisting largely of cells with a basal phenotype (*figure 1.5*). The lamina reticularis is altered in patients with asthma. This region, which is composed of collagen I, collagen III, collagen V, fibronectin and tenascin and is situated just below the basement membrane, has an overall thickness of 3-4 μm in non-asthmatic subjects, whereas in asthma this is increased two- to three-fold (Davies *et al.*, 2003). The damaged epithelium gives rise to further penetration of environmental agents, resulting in further damage and initiating a 'vicious circle' of inflammatory responses (Holgate *et al.*, 2000).

The loss of the bronchial epithelium has a number of consequences that appear to be critical in the exacerbation of asthma. The loss of the epithelial barrier exposes mucosal afferent nerves to non-specific stimulation and allows molecules to penetrate deep into the tissues (Vignola AM et al, 1998); noxious stimuli are harder to remove because the epithelium is unable to carry out sufficient mucociliary clearance (Montefort *et al.*, 1992); loss of epithelial derived relaxant factors, NO and PGE_2, makes smooth muscle more susceptible to stimulation (Folkerts *et al.*, 1998); the epithelium is also a source of

enzymes that are able to degrade proinflammatory neuropeptides, thus the absence of these enzymes would lead to a neural reflex that causes smooth muscle contraction (Vignola AM et al, 1998); disruption in communication between the epithelium and the underlying mesenchymal cells leads to an increased amount of extracellular matrix deposition (collagens I, III & V, fibronectin and tenascin) and it stimulates the proliferation of (myo)fibroblasts, nerve cells, smooth muscle cells and blood vessels (Holgate ST et al, 2000).

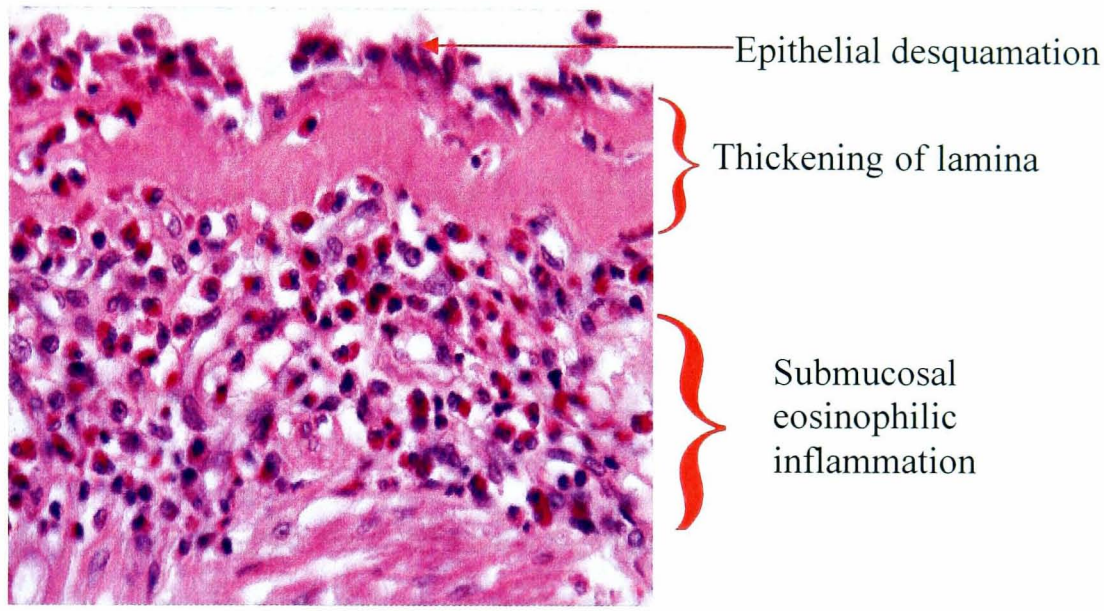

Figure 1.5. Histological section through the bronchial epithelium of an asthmatic airway. The columnar cells have been shed and the bronchial epithelium is denuded to the basement membrane. The section demonstrates airway remodelling, including thickening of the lamina reticularis.

The reason behind the inherent fragility of the asthmatic airway is not fully known. Early studies involved endobronchial biopsies and BAL to obtain direct evidence for bronchial epithelial damage in asthma. Studies involving bronchial biopsies from normal and asthmatic patients demonstrated that the loss in bronchial epithelium was far greater in asthmatic subjects. However, the invasive technique of the bronchoscopy must not be dismissed. One study showed that there was no difference between the asthmatic and normal subjects as both had a large degree of epithelial shedding (Ordonez *et al.*, 2000). On the contrary, studies have shown that the bronchoscopy procedure does result in a certain degree of epithelial shedding in both subjects; however, the asthmatic epithelium is significantly more fragile and appears to be less securely attached to the underlying basement membrane, rendering it more susceptible to injury by the biopsy instruments (Jeffery *et al.*, 1989). One of the first studies by Laitinen *et al* (Laitinen *et al.*, 1985) involved the use of the rigid bronchoscope to obtain

bronchial biopsies from 8 asthmatics and one non-asthmatic subject. The bronchial mucosa showed widespread disruption of the epithelium, loss of cilia and disturbance of alignment of cilial basal bodies but analysis of the non-asthmatic biopsy revealed that the pseudostratified epithelium was intact. In order for the results of this study to be conclusive, there should perhaps have been a larger sample of non-asthmatic controls. however, the results support the argument that epithelial damage in asthma is not due to an artefact. There is little doubt that the airway of asthmatics is abnormal or that it has increased susceptibility to injury compared with the normal epithelium. Tight junctions appear to be a likely target for epithelial injury and since they have a critical role in maintaining epithelial integrity, and are crucial to the regulation of cell permeability, cleavage of these junctions could be of major significance to the pathogenesis of asthma.

1.2.4. Effects of inflammation in asthma: Airway remodelling

While acute inflammation is a beneficial, non-specific response of tissues to injury that generally leads to repair and restoration of normal structure and function, asthma represents a chronic inflammatory process of the airways followed by healing, which characteristically leads to altered structure, referred to as airway remodelling (Beckett *et al.*, 2003). Airway remodelling encompasses both structural and functional consequences of altered airway morphology, however, the factors that initiate and perpetuate remodelling are not fully known.

The presence of AHR implies a fundamental change in the function of airway smooth muscle (ASM) that is partially due to changes in ASM structure and its relationship with the surrounding airway wall structures. Airflow obstruction and AHR are a result of contraction of ASM and hence indicate alterations in smooth muscle function. Several studies have reported an increase in the apparent muscle mass within the airway wall in patients with severe asthma when compared to control subjects (Ebina *et al.*, 1993; Kuwano *et al.*, 1993). Furthermore, necropsy studies suggest that there may be two patterns: type 1, whereby there is increased muscle mass associated with hyperplasia that is restricted to the large central airways; and type 2 which describes the presence of mild hyperplasia in the large airways, but the detection of hypertrophy throughout the bronchial tree, especially in the small peripheral airways. Subjects with

fatal asthma have been reported to exhibit greater degrees of luminal narrowing and ASM mass when compared to patients with asthma whom experienced a non-asthma related death (Ebina *et al.*, 1993). The majority of the available evidence supports ASM hyperplasia as an important mechanism leading to increased ASM mass in the asthmatic airway. Growth factors that bind to receptor tyrosine kinases and ligands that bind to heterotrimeric G protein-coupled receptors have been shown to have the capacity to induce ASM hyperplasia. These agents might be produced by resident airway cells, secreted by infiltrating inflammatory cells, or secreted by ASM itself, which could then act in an autocrine manner (Howarth *et al.*, 2004). Total airways resistance and non-specific AHR are increased in asthma. Potential mechanisms for the presumed ASM hypercontractility could include changes in the balance of contractile-relaxant receptors, changes in signalling, or changes in the actin-myosin force transduction apparatus. Histopathologic studies have demonstrated that the entirety of the airway wall, i.e. the mucosa, submucosa (including ASM) and adventitia is thickened; therefore it is likely that a greater force would need to be generated by the ASM contractile apparatus in the asthmatic airway (Jeffery, 2001). It has been reported that asthmatic ASM has an increased maximal velocity of muscle shortening attributable to an increase in the activity of myosin light chain kinase, which might explain the reduced bronchodilating effect of inspiration in asthmatics. This has been suggested as one of the main causes of AHR (Jiang *et al.*, 1992). Stimulated ASM cells have been shown to be capable of producing a wide range of inflammatory mediators that might contribute to airway remodelling (Howarth *et al.*, 2004; Panettieri, 2003).

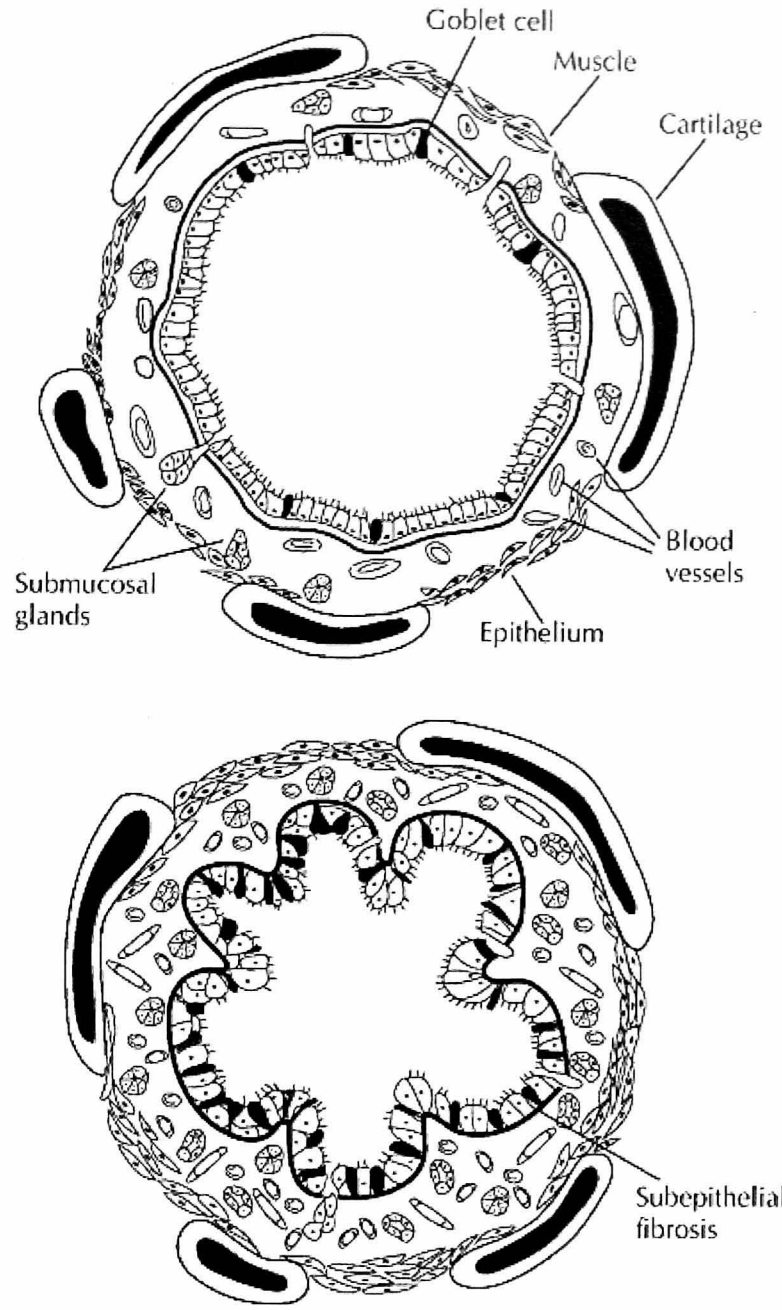

Figure 1.6. A diagrammatic representation of the normal (top) and asthmatic (bottom) airways. The asthmatic airway demonstrates smooth muscle hyperplasia, subepithelial fibrosis, goblet cell hyperplasia and angiogenesis (Fahy *et al.*, 2000).

Thickening of the lamina reticularis below the true basement membrane is a characteristic, early feature of the asthmatic bronchus and is termed subepithelial fibrosis or sub-basement membrane thickening. An approximate two-fold increase in sub-membrane thickness has been reported in asthmatics of varying severity compared to non-asthmatic controls (Hoshino *et al.*, 1998). This thickening is associated with deposition of collagen I, collagen III and fibronectin (Roche *et al.*, 1989). The predominant source of these matrix molecules is thought to be the subepithelial myofibroblast, which is α-smooth muscle actin positive and forms a specialised network beneath the lamina reticularis. Studies have demonstrated a correlation between the thickness of the lamina reticularis and the number of subepithelial myofibroblasts

(Brewster *et al.*, 1990). Furthermore, several profibrotic cytokines, including TGF-β, and PDGF, and mediators such as endothelin-1 can be produced by epithelial cells and macrophages in the inflamed airway (Redington, 2000). MMPs are key regulators of the components of matrix and are involved in the degradation of collagen (Kelly *et al.*, 2003). TIMPs are endogenously expressed and exist in equilibrium with the metalloproteinases in the airway wall. An imbalance between these agents, favouring TIMPs could result in increased matrix deposition and subsequent airway remodelling (Vignola *et al.*, 2004). IL-13 is considered a major cytokine involved in inflammation and remodelling and many of the effects of IL-13 appear to be mediated by matrix metalloproteinases. In one study, the effects of IL-13 over-expression were found to be mediated by TGF-β, whose activation was MMP-9-dependent (Lee *et al.*, 2001). Notably, ADAM33 is a recently identified member of a family of genes encoding a sub-group of the zinc-dependent metalloproteinase superfamily termed the A Disintegrin And Metalloproteinase (ADAM) family and recent epidemiological studies have identified a role for ADAM33 as a susceptibility gene for progressive and severe asthma (Holgate *et al.*, 2006).

An increase in the number and size of blood vessels has been reported in biopsy samples from airways in asthmatic subjects compared with controls (Li *et al.*, 1997). In fatal asthma, there is an increase in the number and size of large blood vessels and a reduction in the number and size of small vessels (Carroll *et al.*, 1997).

The mucosal glands in the segmental bronchi of asthmatics are considerably enlarged, with a volume twice that of non-asthmatic subjects (Dunnill, 1960; Dunnill *et al.*, 1969). Mucous production is an important feature of asthma and contributes substantially to morbidity and mortality, especially in severe asthma. Epithelial goblet cell hyperplasia has been consistently demonstrated in asthma, irrespective of whether the disease is mild or severe. Similarly, enlargement and hyperplasia of mucous glands found in the subepithelium is also common in asthma (Pascual *et al.*, 2005). In addition to mucous-producing cells, the epithelium also consists of basal cells and pseudostratified cells that bear cilia. Basal cells are important for epithelial regeneration and hence its integrity. Ciliated cells move mucous in a general cephalad direction, providing an important mechanism for clearing secreted mucous, particulate matter, allergen particles and infectious agents. In a culture model of primary epithelial cells, IL-13 was shown to

increase the proportion of secretory cells, thereby altering epithelial cell morphology and reduce the number of ciliated epithelial cells (Laoukili *et al.*, 2001).

Asthmatic airway biopsies, autopsy studies and animal models all show that the epithelium is remodelled in asthma. Bronchial epithelial shedding is a characteristic feature of asthma and may be important in contributing to AHR. Epithelial cells appear to be an important source of mediators in allergic inflammation and release of these mediators may be stimulated by various inhaled stimuli, resulting in an increased inflammatory response. Epithelial cells may also release growth factors that stimulate structural changes in the airways, including fibrosis, angiogenesis and proliferation of airway smooth muscle. These responses may be seen as an attempt to repair the damage caused by chronic inflammation (Holgate *et al.*, 2000). Following injury, the epithelium responds by creating the necessary stimuli to recruit the underlying mesenchyme to participate in the repair process. This is mediated through the release of a range of growth factors, which together promote the remodelling and vasculogenesis that is typical in the asthmatic airways. However, in severe cases, the airways exhibit characteristics of a chronic wound with evidence of ongoing epithelial injury and repair. (Holgate *et al.*, 2006).

The historical perspective was that epithelial damage in asthma was induced by the release of cytotoxic factors such as highly basic proteins and reactive oxygen species from activated leukocytes (Erjefalt *et al.*, 1997; Filley *et al.*, 1982; Frigas *et al.*, 1991; Mendis *et al.*, 1990; Venaille *et al.*, 1995; Yukawa *et al.*, 1990). However, the currently favoured model describes a defective repair response or recurrent injury that leads to a self-perpetuating cycle of chronic inflammation, modulated by various cytokines and growth factors. Recent opinions are that exaggerated inflammation and remodelling in the airways are a consequence of abnormal injury and repair responses arising from the susceptibility of the bronchial epithelium to components of the inhaled environment (Davies *et al.*, 2002). This represents a novel concept because up until now, inflammation has been considered the cause of injury and remodelling.

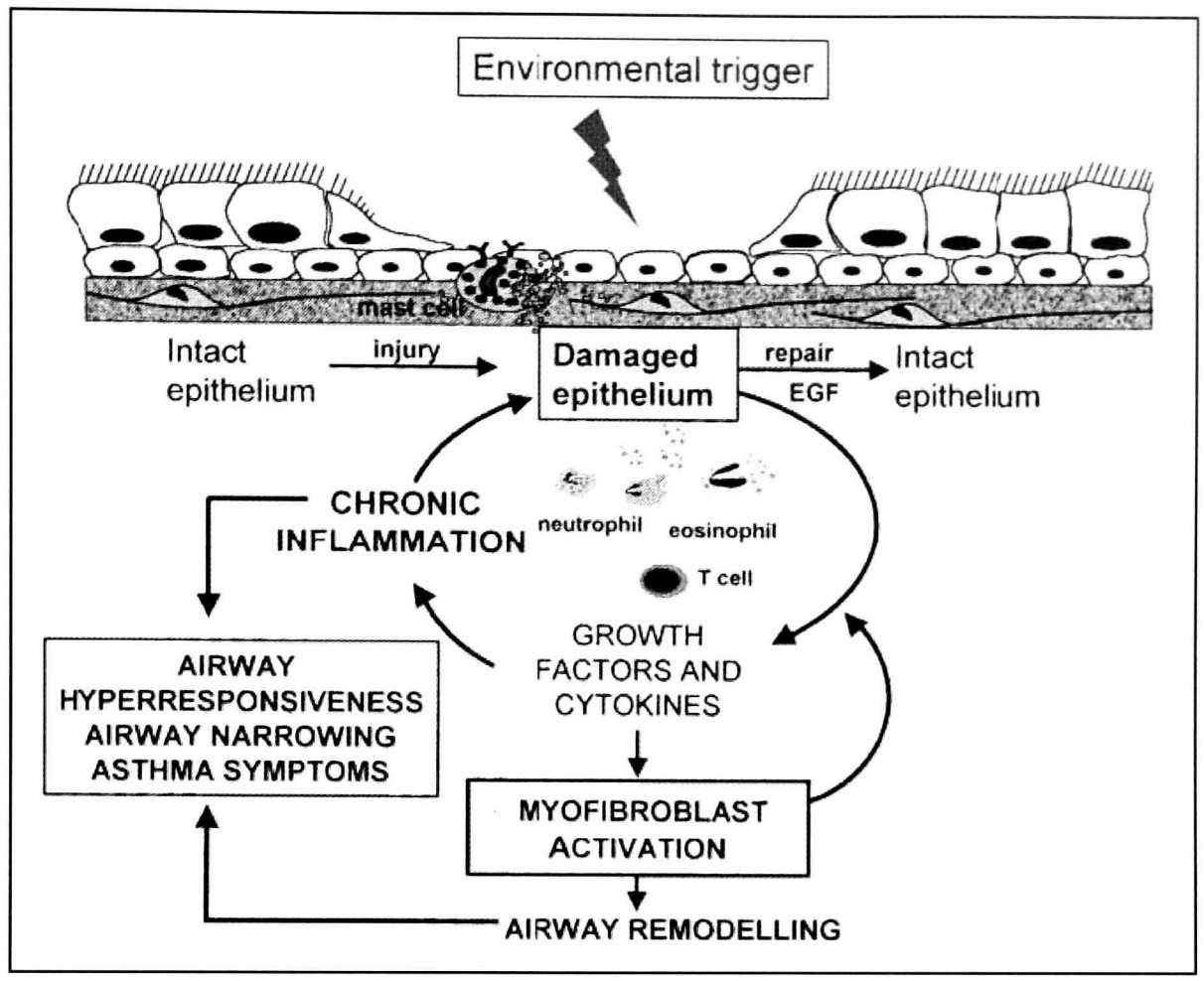

Figure 1.7. A diagrammatic model for the interaction between environmental agents and a susceptible epithelium as a trigger for persistent airway inflammation and remodelling in asthma. Susceptibility to environmental oxidants causes epithelial damage, which triggers an injury-repair response involving release of mediators that promote inflammation and tissue repair. However, the release of endogenous oxidants by inflammatory cells causes further injury to the susceptible epithelium, resulting in a chronic state of tissue damage and thus, persistent inflammation and tissue remodelling (Holgate *et al.*, 2000).

In asthma, the bronchial epithelium is considered to be defective (Holtzman *et al.*, 2002) and there is accumulating evidence to support this. It has been demonstrated that the transcription factor STAT1 is constitutively activated in epithelial cells from asthmatic subjects compared with non-asthmatic controls. STAT1 is a key transcription factor in interferon (IFN)-mediated signalling, which is an important mechanism in viral airway infection, a known cause of asthma exacerbation. Epithelial STAT1 levels correlated with intercellular adhesion molecule 1 expression, which in turn correlated with T-lymphocyte accumulation in asthmatic tissue (Sampath *et al.*, 1999).

The bronchial epithelium in asthma has been shown to be unable to effectively defend the lung against human rhinovirus, which is normally considered an innocuous virus. The normal response of the epithelium to viral infection is the induction of primary

IFNs, such as IFN-β, through activation of the Toll-like receptor 3, which recognises double-stranded viral RNA, leading to apoptosis and subsequent elimination of the infected cell and thus, limitation of viral infection and release. However, in asthmatic epithelial cells, a major defect in this pathway leads to enhanced viral replication and virus-induced cell toxicity. Furthermore, decreased expression of IFN-β and IFN-γ have been reported in asthma (Holgate, 2006). An alternative explanation is that tight junctions are defective in asthma, resulting in increased susceptibility to infection.

When epithelial surfaces are injured, the normal response is upregulation of receptor expression, specifically of members of the EGFR family that are responsible for proliferation and repair. Biopsies from patients with asthma demonstrate a marked increase in the expression of the EGFR, whose expression increases in proportion to disease severity and chronicity (Amishima *et al.*, 1998; Puddicombe *et al.*, 2000). EGFR immunostaining in severe asthma is demonstrated throughout the repairing epithelium and also on the luminal surface, a feature not observed in the normal epithelium. However, contrary to expectations, the high level of EGFR expression in the asthmatic epithelium is not paralleled by an increase in markers of cell proliferation (Demoly *et al.*, 1994). In mild asthma, there is also marked cytoplasmic staining of columnar cells for $p21^{waf}$, indicating an attempt to protect the surface epithelial cells from premature program cell death by inhibiting the cell cycle at the G1 phase. However, in severe disease, $p21^{waf}$ is found throughout the epithelium and specifically at a nuclear location, where it impairs proliferation by arresting cells at the G1 phase of the cell cycle (Puddicombe *et al.*, 2003). This negative regulator of G1 cyclins can be induced by the antiproliferative TGF-β1 and TGF-β2, the levels of which are increased in patients with asthma (Redington *et al.*, 1997). In addition, the EGFR might contribute directly to inflammation because EGF and TGF-α are known to elicit IL-8 expression and release from bronchial epithelial cells. Since the EGFR is over-expressed in the morphologically intact epithelium in severe asthma and strongly correlates with IL-8 expression, this may contribute to airway neutrophilia in patients with severe asthma (Hamilton *et al.*, 2003).

Taken together, these findings lead to the suggestion that in chronic asthma the airway epithelium is impaired in its ability to restitute itself after injury and, as a consequence, enters into a chronic wound response. In order to understand the mechanisms involved

in the repair of the asthmatic epithelium in response to injury, it is imperative to determine the mechanisms of normal bronchial epithelial repair.

1.3. Hypothesis of study

The formation of fibrin to support bronchial epithelial repair *in vitro* is a result of the local release of coagulation factors from bronchial epithelial cells and is independent of plasma proteins.

1.3.1. Aims of study

The initial aim of the study was to establish whether the bronchial epithelium had the capacity to generate coagulation factors in the absence of plasma. Subsequent aims were to demonstrate the involvement of locally expressed coagulation factors in wound repair. Finally, it was imperative to identify the mechanisms involved in wound repair by incorporating pharmacological agents that manipulate the wound repair process.

Chapter 2.

Materials & General Cell Culture Methods

2. Materials and general cell culture methods

2.1. Materials

2.1.1. Cell culture

Cells of the human bronchial epithelial cell line, 16HBE 14o$^-$, were a gift from Dr Dieter Gruenert (University of Vermont, Burlington, VT).

Foetal bovine serum (FBS) heat inactivated, sterile filtered; 100X Insulin-transferrin-sodium selenite (ITS) media supplement, Tween-20, Triton-X100, collagen IV and albumin from bovine serum (BSA) were from Sigma (Dorset, UK).

Minimal essential medium (MEM) (1X) containing Earle's Salts and L-glutamine, 10X phosphate-buffered saline (PBS) without calcium and magnesium, 10X trypsin-EDTA (0.5% ($^w/_v$) trypsin, 5.3mM EDTA), antibiotic/antimycotic (penicillin G 100 U/ml, streptomycin 100 µg/ml, amphotericin B 25 µg/ml), L-glutamine, HBSS and 0.4% trypan blue, were from Invitrogen Ltd (Paisley, UK).

Normal human bronchial epithelial (NHBE) cells in bronchial epithelial growth medium (BEGM) with retinoic acid (<1 µg/ml) and BEGM BulletKit® were from Cambrex BioScience Wokingham Ltd (Wokingham, UK).

Nunc 6-well plates and Nunc Maxisorp® 96-well plates were from Fisher Scientific (Leicestershire, UK).

24-well plates and 75 cm^2 tissue culture flasks were from Triple Red Laboratory Technology (Buckinghamshire, UK).

2.1.2. General materials

Acetic acid, ethanol, hydrochloric acid, methanol, sodium hydroxide, sulphuric acid, sodium chloride, sodium dodecylsulphate (SDS) and Tris were from Fisher Scientific (Leicestershire, UK).

Biotinylated molecular weight ladder, HEPES, HGF, hydrogen peroxide, lactate dehydrogenase (LDH) kit: Tox-7, mouse IgG, rabbit IgG, sheep IgG, neutrophil elastase, tetramethylbenzidine (TMB), indomethacin, PGE_2, DMSO, mitomycin C, sodium deoxycholate, ethidium bromide, sodium butyrate and trichostatin A were from Sigma (Dorset, UK).

Factor Xa, DTSSP and protease cocktail inhibitor were from Calbiochem (supplied by Merck Chemicals Ltd; Nottingham, UK).

Goat anti-mouse F(ab') fragments, biotinylated rabbit anti-mouse $F(ab')_2$ fragments and streptavidin-biotin complex (StreptABComplex) were from Dako (Cambridgeshire, UK).

Goat anti-mouse Alexa Fluor 488 $F(ab')_2$ fragment and donkey anti-sheep Alexa Fluor 594 were from Molecular Probes (supplied by Invitrogen Ltd; Paisley, UK).

Human EGF, KGF, TGF-β1, HGF and PGE_2 immunoassays were from R&D Systems (Oxfordshire, UK).

IL-8 ELISA kit was from Sanquin Reagents (Amsterdam, The Netherlands).

Loading dye, dNTP, random hexamer, 5X concentrated first strand buffer, RNaseOUT inhibitor, Superscript II reverse transcriptase, One-Step RT-PCR system, 100 base pair ladder and DTT were from Invitrogen Ltd (Paisley, UK).

Mastermix was from Qiagen (West Sussex, UK).

Monoclonal mouse anti-human TF antibody, mouse anti-human D-dimer antibody and high molecular weight (HMW) D-dimer standard were from American Diagnostica (Stamford, CT, USA (supplied by Axis-shield, Dundee, UK)).

Nitrocellulose membrane was from Bio-Rad Laboratories (Hertfordshire, UK).

Normal reference plasma was from Alpha Laboratories Ltd (Hampshire, UK).

PAR-1 peptide agonist TFRIFD-amide, PAR-1 control peptide agonist FTRIFD-amide, PAR-2 peptide agonist SLIGKVD-amide and PAR-2 control peptide agonist LSIGKVD-amide were from Peptide Protein Research Ltd (Hampshire, UK).

Paraformaldehyde, agarose and EDTA were from BDH (Dorset, UK).

Primer sets for 18SRNA, β-actin, TF, FVII, FGC, FX, FXIII, PAR-1, PAR-2, EP-1, EP-2, EP-3 and EP-4 were from Sigma-Genosys Ltd (Cambridge, UK).

Prothromplex TIM-4 was from Baxter Healthcare (Norfolk, UK).

RNAgents® denaturing solution, sodium acetate, phenol:chloroform:isoamyl alcohol, isopropanol and nuclease-free water were from Promega (Hampshire, UK).

S-2222 (Factor X substrate) was from Chromogenix (Milan, Italy (Supplied by Quadratech; Surrey, UK)).

Sheep anti-human FXIIIA-HRP IgG, sheep anti-human fibrinopeptide A, affinity purified, IgG and sheep anti-human fibrinopeptide A-HRP IgG were from Affinity Biologicals (Ontario, Canada (supplied by Quadratech; Surrey, UK)).

SuperSignal® West Pico Substrate and Seize® X protein A immunoprecipitation kit were from Pierce (Supplied by Perbio; Northumberland, UK).

Thrombin inhibitor UK-156,406 and FXa inhibitors UK-220,047 and PD-031 were from Pfizer Global R&D (Kent, UK).

Vectashield mounting medium was from Vector Laboratories Ltd (Peterborough, UK).

2.2. General cell culture methods

2.2.1. Cell culture of 16HBE 14o$^-$ cells

All cell culture work was carried out in a class II laminar flow tissue culture hood. The 16HBE 14o$^-$ bronchial epithelial cell line was a kind gift from Dr Dieter Gruenert (University of Vermont, Burlington, VT). Stock cultures were originally seeded at a density of 3500 cells/cm^2 in 75 cm^2 tissue culture grade flasks and routinely maintained in full minimal essential media (MEM) with Earle's salts, supplemented with 10% ($^v/_v$) heat inactivated FBS, 2 mM L-glutamine and 100 U/ml antibiotic/antimycotic at 37°C in a 5% CO_2 incubator.

At confluence, cells were washed in 1X PBS pH 7.2, without calcium and magnesium. Cells were passaged by adding 3 ml of 1X trypsin-EDTA (derived from 10X stock solution containing: 0.5% trypsin, 5.3 mM EDTA) to each flask for 10 minutes at 37°C. As a consequence, the cells became rounded and subsequently lifted from the flask into suspension. Once lifted, 2 ml of FBS was added to each flask of cells to neutralise trypsin activity. The cell suspension was centrifuged for 7 minutes at 670 x *g* (ALC PK120 Centrifuge, Winchester Virginia, USA) to create a cell pellet. The supernatant was discarded and the pellet resuspended in 1 ml full MEM. For determination of cell number, the cell suspension was diluted 1 in 10 with trypan blue and 10 µl was added to each grid of a haemocytometer. Cells were counted within the 5 x 5 grid and an average cell count was calculated for the two grids. The number of cells required for the appropriate cell seeding density was then calculated (*figure 2.1*).

$$\text{No. cells/ml} = \text{Cell count} \times 10^{*} \times 10^{4\,**}$$

* Dilution factor of cells in trypan blue

** Dilution factor of haemocytometer

Figure 2.1. The Equation used to calculate the number of cells required for cell seeding.

Cells were seeded into 24-well plates at 100,000 cells per well and cultured for 48 h until fully confluent. At each passage, any remaining cells were transferred into new culture flasks at a seeding density of 3500 cells/cm^2 to allow a continuous supply. At confluence, cells were washed by adding 500 µl of 1X PBS (without calcium and magnesium) to each well and quiesced for 16 h in 250 µl per well of serum-free MEM-ITS (basal MEM containing 2 mM L-glutamine and 100 U/ml antibiotic/antimycotic, supplemented with 1% ($^{v}/_{v}$) Insulin Transferrin Selenium solution). Immediately prior to each experiment, cells were washed by adding 500 µl of 1X PBS to each well and the serum-free MEM-ITS was refreshed by adding 250 µl to each well.

2.2.2. Collagen coating of flasks for NHBE cell culture

5 ml of 0.1 mg/ml collagen IV in 3% ($^{v}/_{v}$) acetic acid was added to each flask and incubated for one hour at room temperature. Excess collagen IV was removed and flasks were washed with 5 ml of 1X Hanks' balanced salt solution (HBSS) (with phenol red and without calcium and magnesium). The wash step was repeated 3 times, until there was no further colour change of the phenol red, indicating complete removal of the acetic acid.

2.2.3. Cell culture of NHBE cells

NHBE cells in BEGM with retinoic acid (<1 µg/ml) were purchased from Cambrex Bio Science Wokingham, LTD (Wokingham, UK). Stock cultures were originally seeded at a density of 3500 cells/cm^2 on 10 µg/cm^2 collagen IV-coated 75cm^2 flasks and were

cultured in BEGM (BulletKit®: Cambrex, Wokingham, UK), supplemented with 1% ($^{v}/_{v}$) heat inactivated FBS at 37°C in a 5% CO_2 incubator. At confluence, cells were washed in 1X PBS pH 7.2 without calcium and magnesium. Cells were passaged by adding 3 ml of 0.5X trypsin-EDTA (derived from 10X stock solution containing: 0.5% trypsin, 5.3 mM EDTA) to each flask for 10 minutes at 37°C. As a consequence, the cells became rounded and subsequently lifted from the flask into suspension. Once lifted, 2 ml of FBS was added to each flask of cells to neutralise trypsin activity. The cell suspension was centrifuged for 7 minutes at 670 x *g* (ALC PK120 Centrifuge, Winchester Virginia, USA) to create a cell pellet. The supernatant was discarded and the pellet resuspended in 1 ml full BEGM. For determination of cell number, the cell suspension was diluted 1 in 10 with trypan blue and 10 µl was added to each grid of a haemocytometer. Cells were counted within the 5 x 5 grid and an average cell count was calculated between the two grids. The number of cells required for the appropriate cell seeding density was then calculated (*figure 2.1*).

Cells were seeded into 10 µg/cm^2 collagen IV-coated 24-well plates at 100,000 cells per well and cultured for 5 days until fully confluent. At each passage, any remaining cells were transferred into new collagen IV-coated culture flasks to allow a continuous supply. NHBE cells were used with a maximum of 3 passages. At confluence, cells were washed by adding 500 µl of 1X PBS (without calcium and magnesium) to each well and quiesced for 16 hours in 250 µl of serum-free BEGM-ITS (basal BEGM containing 2 mM L-glutamine and 100 U/ml antibiotic/antimycotic, supplemented with 1% ($^{v}/_{v}$) ITS). Immediately prior to each experiment, cells were washed by adding 500 µl of 1X PBS to each well and BEGM-ITS was refreshed by adding 250 µl to each well.

2.2.4. Harvesting of cells and cell culture supernatants

Following mechanical wounding and subsequent incubation at 37°C, supernatants and cells were harvested and either analysed immediately or stored for future use. Supernatants were removed from cells into 1.5 ml centrifuge tubes and centrifuged at 900 x *g* for 10 minutes at 4°C (ALC PM140R Centrifuge, Winchester Virginia, USA) to remove cell debris. The resultant, cleared supernatants were transferred into new 1.5 ml centrifuge tubes and stored at -80°C if not required immediately. The cells in the plate

were washed with 500 µl per well of ice-cold, 1X PBS, and 250 µl of ice-cold lysis buffer (10mM Tris-HCl pH 7.4, 0.1mM $CaCl_2$, 0.1% ($^{w}/_{v}$) BSA and 1% ($^{v}/_{v}$) Triton-X-100) was added to each well. Once the cells had lifted, the cell lysates were extracted from the plate into 1.5 ml centrifuge tubes and centrifuged at 900 x *g* for 10 minutes at 4°C (ALC PM140R Centrifuge, Winchester Virginia, USA) to remove cell debris. The resultant, cleared cell lysates were transferred into new 1.5 ml tubes and stored at -80°C if not required immediately.

2.2.5. Mechanical wounding

To induce mechanical damage, 16HBE 14o^{-} cells were grown to confluence in serum-free MEM-ITS on 24-well plates and were mechanically wounded by scraping off a line of cells in a cross-hatch pattern using a P2 Gilson pipette tip as shown below:

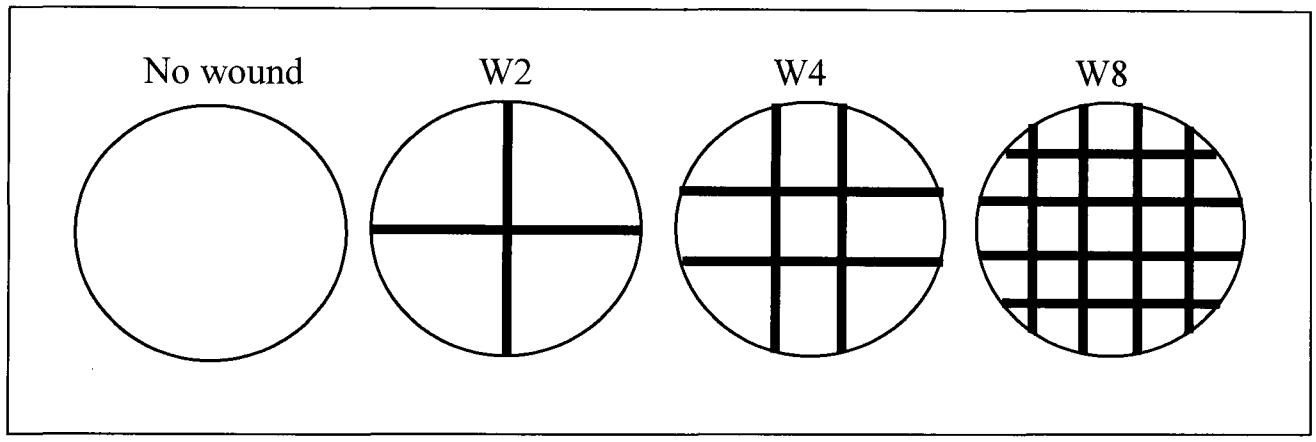

Figure 2.2. Schematic representation of mechanical wounding. Black circle represents one cell of a 24-well plate; straight line denotes one mechanical wound, as described above. W2 = 2 mechanical wounds, W4 = 4 mechanical wounds and W8 = 8 mechanical wounds. Width of mechanical wound immediately after wounding measures approximately 0.5 mm. Diagram not to scale.

2.2.6. Data analysis

In all cases, statistical analysis was performed using a two-tailed unpaired Student's *t*-test using GraphPad InStat software and are represented as the mean ± SEM. A two-tailed p value of less than 0.05 was considered statistically significant.

Figures representing *n=3* are indicative of the mean of duplicate conditions within the experiment, performed on three separate occasions.

Chapter 3.

Coagulation Factor Expression & Role of Inflammatory Mediators

3. Coagulation and growth factor expression in response to wounding and role of inflammatory mediators

3.1. Introduction

3.1.1. Role of plasma derived coagulation factors in bronchial epithelial repair

Many investigations have demonstrated that following the removal of the guinea pig bronchial epithelium *in vivo,* there is a rapid process of repair, whereby epithelial cells at the edge of the wound flatten and migrate to cover the denuded area, then proliferate and differentiate (Erjefalt *et al.*, 1997; Persson *et al.*, 1998). It has been suggested that a fibrin gel matrix is required to facilitate cell migration (Erjefalt *et al.*, 1994). In the airways, there is a profuse network of microvessels directly beneath the pseudostratified epithelium. Following epithelial damage and subsequent shedding, to the point of denudation, several tissue responses are initiated in order to minimise the harmful effects of an impaired epithelial barrier and ultimately restore the integrity of the bronchial epithelium. The mucosal microcirculation constitutes an important repair- and defence-promoting component during the initial repair phases. In guinea pig models of epithelial damage, interendothelial permeability pores are formed in subepithelial venules within minutes of epithelial shedding (Erjefalt *et al.*, 1996); and a pronounced, local plasma exudation response is initiated (Erjefalt *et al.*, 1994). Extravasated bulk plasma immediately enters the airway lumen and a gel-like network of fibrin and fibronectin fibres and other associated plasma proteins, is formed on the denuded basement membrane (Erjefalt *et al.*, 1994). *In vivo* experiments involving wholemount three-dimensional morphology have demonstrated the presence of small round holes along short stretches of venules of the airway mucosa, correlating to increased vascular permeability (Erjefalt *et al.*, 1996). Subsequently, it has been shown that non-sieved plasma flows through the gaps into extravascular sites by a hydrostatic pressure gradient (Grega *et al.*, 1988). During the first 10-20 seconds after epithelial damage, the lamina propria is flooded with the plasma exudate, which then passes freely through the epithelial basement membrane and up between epithelial cells. Interestingly, the bulk plasma exudate has the capacity to flow through the tight junctions of the intact bronchial epithelium and into the airway lumen (Persson *et al.*, 1986). One of the

predominant roles of the plasma exudate is to provide the coagulation factors that will form the fibrin gel matrix that is required for bronchial epithelial repair. The formation of fibrin, termed fibrinogenesis ultimately results from the initiation of the extrinsic coagulation cascade.

3.1.2. The extrinsic coagulation cascade

The extrinsic coagulation cascade is initiated upon damage to the bronchial epithelium. This occurs by contact of plasma proteins with active TF at sites of extravascular injury. TF acts as a receptor for FVII, which is converted to active FVIIa by cleavage of a single peptide bond (*figure 3.1*) (Morrissey, 2001). This complex then activates FX which, together with FVa, is one of the main components of the prothrombinase complex, responsible for the conversion of prothrombin to active thrombin (Dabbagh *et al.*, 1998). Thrombin in turn converts soluble fibrinogen to insoluble fibrin. Thrombin also activates FXIII to FXIIIa, which stabilises the fibrin clot in the presence of calcium *via* covalent cross-links between fibrin molecules and *via* cross-linking of plasmin inhibitor to fibrin (Sidelmann *et al.*, 2000). The presence of fibrin at sites of injury is tightly regulated by the actions of initiators and inhibitors of the coagulation cascade, in addition to the fibrinolytic pathway, which is responsible for the degradation of the clot.

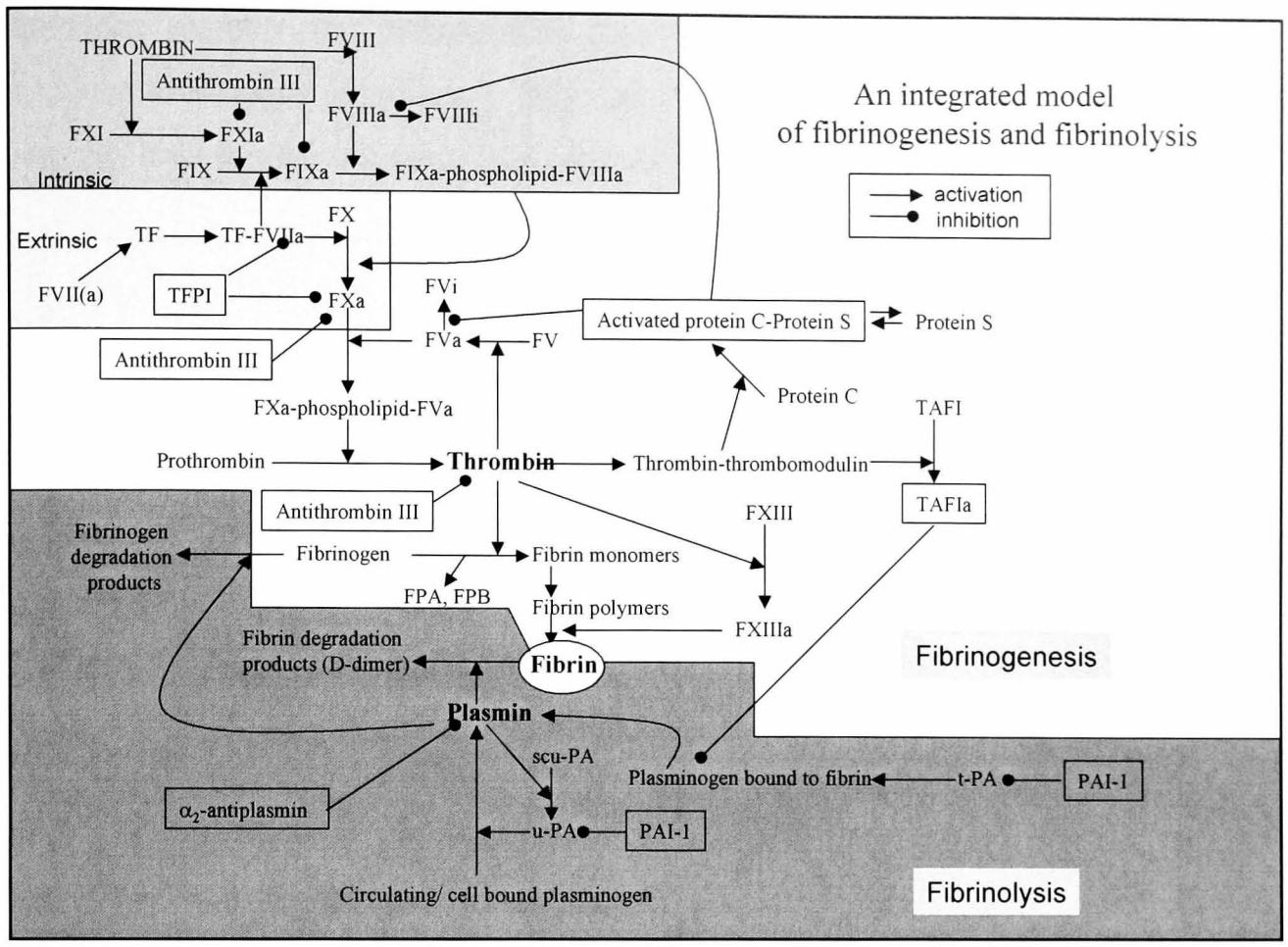

Figure 3.1. A schematic diagram of the processes of fibrin formation (fibrinogenesis) and fibrin degradation (fibrinolysis). Adapted from the review by Sidelmann et al. (2000).

3.1.3. Mediators of fibrinogenesis

3.1.3.1. TF

TF is an integral membrane glycoprotein of 295 amino acids, containing four N-linked glycosylation sites in an organised extracellular sequence of: 219 amino acids, a 23 amino acid hydrophobic transmembrane region and a 21 amino acid cytoplasmic tail (Bazan, 1990). The extracellular domain is made up of two fibronectin type III domains, TF1 and TF2 and has been shown to function as the receptor for FVII (Ruf *et al.*, 1994). TF is a lipoprotein and requires both a protein and a phospholipid portion for full procoagulant activity (Hvatum *et al.*, 1969; Nemerson, 1968). Membrane anchoring of TF is essential to support full procoagulant activity by FVIIa (Paborsky *et al.*, 1991). TF has been classified on the basis of distant sequence homology as a member of the cytokine receptor family (Bazan, 1990). The mobility of TF on SDS gels suggests a molecular weight of 47,000 Da (Bjorklid *et al.*, 1973; Broze *et al.*, 1985). TF is expressed at sites that are physically separated from the circulating blood. This

'envelope' pattern of TF expression signifies that its activation may only occur in response to vascular injury. It is believed that TF is constitutively expressed by cells that play a vital role in repair following injury (Osterud, 1997).

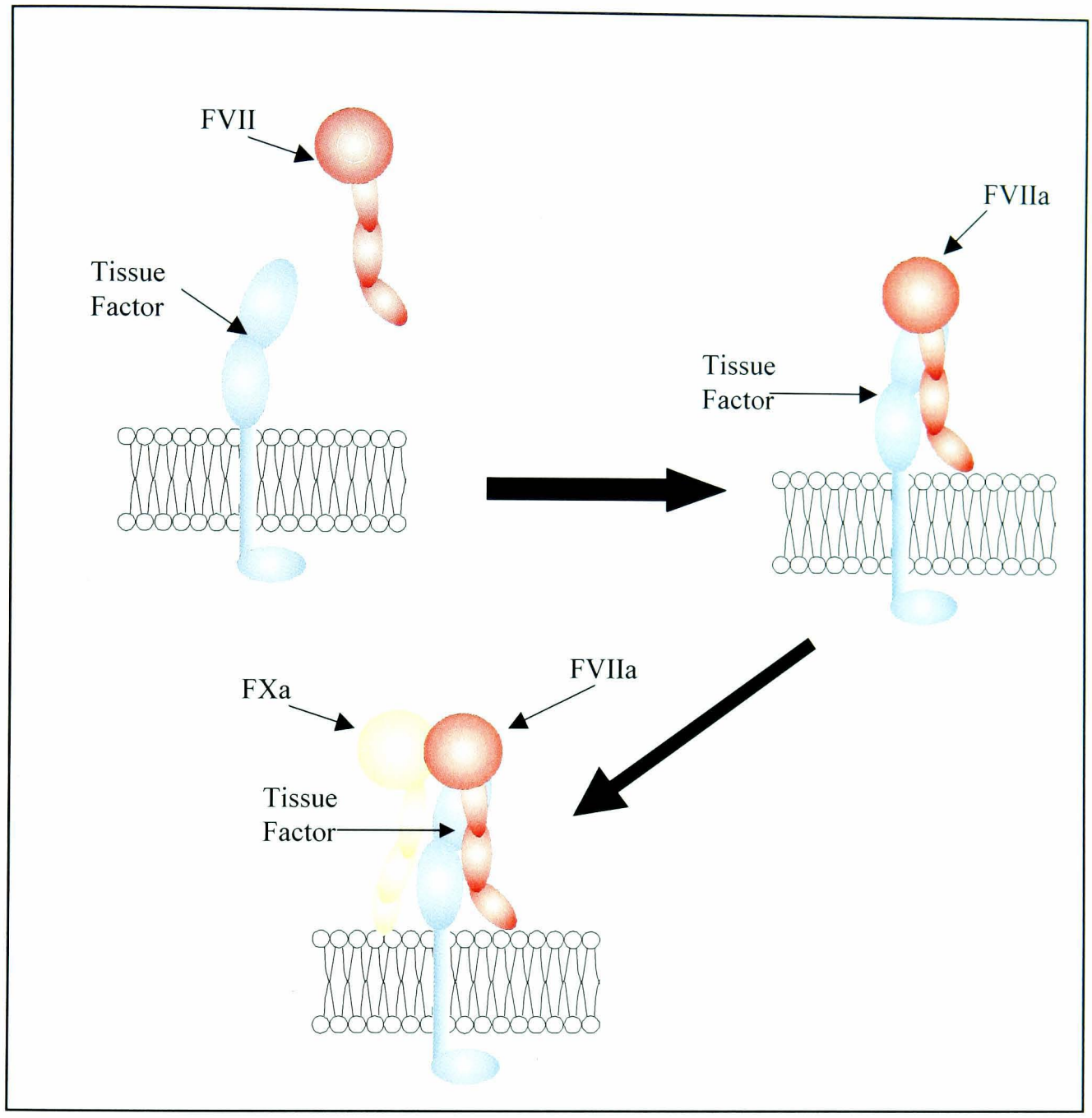

Figure 3.2. Formation of the TF:FVIIa:FXa complex to initiate the coagulation cascade. Transmembranous TF acts as a receptor for circulating FVII, which in turn, binds and activates FX. This complex can also activate PARs and induce signalling through the cytoplasmic domain of TF. Once activated, FXa is released and forms a prothrombinase complex to initiate fibrin formation. FXa may also activate PARs.

The concept of TF encryption has been widely studied in cell culture and may represent a physiological mechanism for controlling expression of cellular coagulant activity *in vivo*. TF encryption describes the post-translational suppression of TF procoagulant activity on the cell surface. An intact endothelium is crucial in the prevention of TF-initiated coagulation *in vivo*; however, there is evidence of TF in normal blood (Giesen

et al., 1999; Key *et al.*, 1998). Thus, there is emerging evidence that TF in normal blood that is associated with monocytes and platelets is encrypted and that encryption may therefore be the primary mechanism controlling the expression of TF procoagulant activity by these cells (Bach, 2006). The first observations of TF encryption were demonstrated in cell culture studies. Unperturbed cells were shown to express very limited TF procoagulant activity despite the fact that TF, an integral membrane protein is localised to the cell surface where it can bind FVII. It was reported that a stimulus was required to express the latent proteolytic activity of the encrypted TF-FVIIa complex. However, a number of factors to induce cells to express encrypted TF procoagulant activity were established, including freezing and thawing, sonication, proteases, phospholipases, non-ionic detergents, apoptosis, complement and calcium ionophores (Bach *et al.*, 1990; Le *et al.*, 1992). The first step in the calcium ionophore-induced decryption of TF procoagulant activity is the release of calcium from internal stores followed by a second and greater influx of extracellular calcium across the plasma membrane. In the plasma membrane of quiescent cells, phosphatidylserine, a major phospholipid, is localised on the inner leaflet of the bilayer (Zwaal *et al.*, 1997); and an increase in cytosolic calcium of the same magnitude that decrypts TF procoagulant activity also disrupts phosphatidylserine asymmetry. The relevance of phosphatidylserine availability is that this phospholipid accelerates coagulation reactions on membrane surfaces (Hathcock *et al.*, 2005; Kunzelmann-Marche *et al.*, 2000). Stimulating cells with a calcium ionophore subsequently alters phosphatidylserine asymmetry and increases the V_{max} of the TF-FVIIa reaction. However, phosphatidylserine asymmetry may not be all that is required for TF encryption *in vivo*. Another possible contribution is suggested by the evidence of an association between TF and lipid rafts (Del Conde *et al.*, 2005; Dietzen *et al.*, 2004). It is not known where encrypted and decrypted TF reside in the plasma membrane, however, it is believed that encrypted TF is localised in lipid rafts and decrypted TF is released into the liquid disordered phase of the membrane following cell stimulation (Bach, 2006). TF procoagulant activity is expressed by decrypted TF-FVIIa, therefore proteolytic activation of FVII must occur at some point in the decryption process. Thus, *in vivo*, the suppressed proteolytic activity of encrypted intravascular TF-FVIIa may explain why infusions of FVIIIa do not trigger thrombosis.

Cell surface TF binds FVII to initiate coagulation, or alternatively, to trigger signalling *via* PAR-2. Recent evidence has emerged that TF-FVII-mediated coagulation and cell signalling involve distinct cellular pools of TF, of which the surface-accessible extracellular disulphide bond is critical for coagulation and protein disulphide isomerase (PDI) disables coagulation by targeting the disulphide bond (Ahamed *et al.*, 2006). Ahamed *et al.* demonstrated that under conditions that favoured TF-FVIIa signalling and disabled coagulant activity, PDI associates with TF on the cell surface, whilst inhibition of PDI expression or activity increased TF coagulant activity. TF-FVIIa signalling *via* PAR-2 contributes to inflammation in the airway; however, Ahamed *et al.* demonstrate that TF-FVIIa signalling can be specifically inhibited without compromising TF-dependent coagulation.

3.1.3.2. FVII

FVII is a glycosylated plasma protein consisting of a single polypeptide chain of 406 amino acids and an overall molecular weight of 50,000 Da (Broze *et al.*, 1980; Kisiel *et al.*, 1975; Radcliffe *et al.*, 1975). FVII is synthesised by the liver and circulates in the plasma at about 500 ng/ml (10nM) (Fair, 1983), however, the free circulating enzyme has very low activity and only attains full activity when in complex with its cofactor TF (Kemball-Cook *et al.*, 1999). FVII is a multi-domain protein, consisting of a gamma-carboxyglutamic acid-rich domain (Gla domain); a hydrophobic or aromatic stack, two EGF-like domains and a serine protease domain that is homologous to trypsin (Morrissey, 2001). The Gla domain confers the ability of FVII to bind to membranes containing negatively charged phospholipids. Membrane binding is calcium-dependent and the crystal structure of the TF-FVIIa complex shows that the Gla domain contains seven bound calcium ions (Banner *et al.*, 1996). FVII shares the same domain structure as factors IX, X and protein C and has a similar gene organisation (Hagen *et al.*, 1986; O'Hara *et al.*, 1987). FVII is converted to the enzymatically competent form, FVIIa *via* proteolysis of a single peptide bond between residues Arg152L (light chain) and Ile153H (heavy chain) (Kemball-Cook *et al.*, 1999). FVII is a single-chain protein, whilst FVIIa is composed of two polypeptide chains, held together by a disulphide bond. The light chain of FVIIa, consisting of the Gla domain, aromatic stack and both EGF domains comprises 152 amino acids and has a molecular weight of approximately 20,000 Da; and the heavy chain, consisting of the serine protease domain, has 254

amino acids and a molecular weight of approximately 30,000 Da (Morrissey, 2001). FVIIa is a highly elongated protein, forming extended contacts with TF. The main sites of contact on FVIIa for TF are located in the first EGF domain and the protease domain, with additional points of contact involving the aromatic stack/Gla region and the second EGF domain. Furthermore, the main sites of contact on TF for FVIIa are located on both fibronectin III domains and the interfacial region between them (Chang *et al.*, 1995; Edgington *et al.*, 1997; Martin *et al.*, 1995). In addition to the enhancement of FVII activity, it has been suggested that TF may play other roles. Firstly, TF may act to locate the active site of FVIIa at the appropriate distance from the membrane to allow it to cleave the macromolecular substrate FX (McCallum *et al.*, 1996; McCallum *et al.*, 1997). There is also evidence that binding of TF to FVIIa alters the FVIIa active site conformation, and the protease domain has been suggested to exist in equilibrium between 'zymogen-like' and fully active forms (Kemball-Cook *et al.*, 1999). The TF-FVIIa complex has extremely restricted substrate specificity and will only bind factors VII, IX and X. However, under most *in vitro* conditions, FX is the preferred substrate (Komiyama *et al.*, 1990).

3.1.3.3. FXa

The serine protease FXa plays a central role in the coagulation cascade, linking the extrinsic and intrinsic pathways by catalysing the conversion of prothrombin to thrombin (Davie *et al.*, 1991). FX associates with the TF-FVIIa complex by interacting with various domains on both proteins (Kirchhofer *et al.*, 2000). In order for FXa activation, calcium is required to promote the binding of enzyme and substrate and subsequent catalysis requires a phospholipid membrane (Nesheim *et al.*, 1979; Rodgers *et al.*, 1983). The γ-carboxyglutamic acid (Gla) domain of FX interacts with phospholipid membrane as well as the C-terminal region of TF and the Gla domain of FVIIa (Ruf *et al.*, 1998).The conversion of FX to FXa results in a reduced affinity for the TF-FVIIa complex and it can be released to form the prothrombinase complex, consisting of phospholipid bound FXa and the cofactor FVa which converts prothrombin to thrombin (Riewald *et al.*, 2002).

3.1.3.4. Thrombin

Thrombin is a trypsin-like member of the chymotrypsin family of serine proteases and is the product of prothrombin cleavage by FXa. Prothrombin is synthesised in the liver as a pre-propeptide and undergoes a number of post-translational processes prior to secretion. Following damage to the epithelium and subsequent initiation of the coagulation cascade, circulating prothrombin is converted to active thrombin. This proteolytic activation is mediated by the prothrombinase complex consisting of prothrombin, FXa, FVa and negatively charged phospholipids, in the presence of calcium ions. During its activation by FXa, prothrombin is cleaved; firstly to generate meizothrombin and then to generate thrombin and fragments that contain the Gla and kringle domains, which function to localize prothrombin onto membrane surfaces to form part of the prothrombinase complex, that includes FVa and FXa (*figure 3.3*). Once prothrombin is activated, thrombin can escape the complex and is free to bind to its substrates. Prothrombin is unique among the activated coagulation proteases in that it completely loses the domains important for initial recognition interactions when it is activated to its serine protease derivative, thrombin. Loss of these domains allows thrombin to diffuse freely to encounter, recognize, cleave, and dissociate from its substrates (Lane *et al.*, 2005). Furthermore, loss of the domains permits exposure of cryptic functional regions (Wu *et al.*, 1994), the active site, and the charged anion binding regions, termed exosites (Bode *et al.*, 1992) which are crucial for extending the range and specificity of the actions of thrombin.

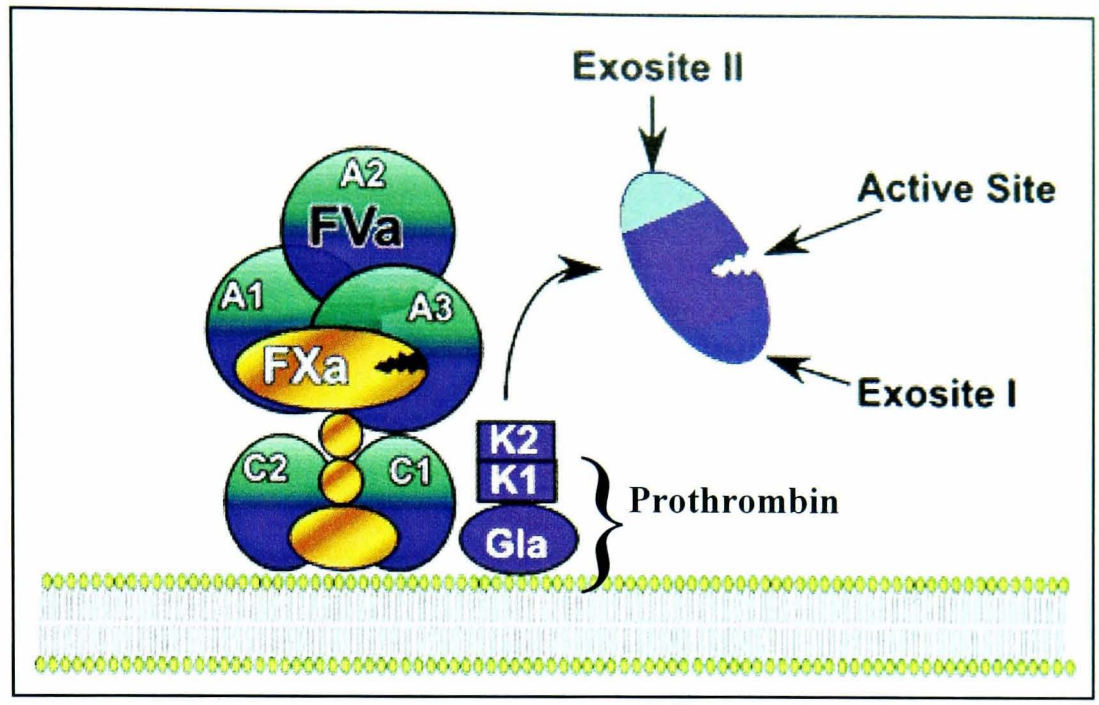

Figure 3.3. Prothrombin activation to thrombin.
Prothrombin is first cleaved and activated as part of the prothrombinase complex to form meizothrombin. The Gla and kringle (K1 and K2) domains are then cleaved, allowing thrombin to diffuse away. A1-A2 = FVa domain fragments that are non-covalently bonded to A3-C1-C2 FVa domain fragments. Thrombin has 3 regions that are functionally inaccessible in prothrombin and become exposed on activation: the active site, exosite I, and exosite II (Lane *et al.*, 2005).

The resultant 39 KDa thrombin molecule comprises two chains, which are cross-linked by four disulphide bonds, that surround a narrow groove containing the catalytic triad consisting of His-57, Asp-102 and Ser-205. This groove is hydrophobic and exhibits a preference for apolar amino acids preceding arginine at a thrombin susceptible bond such as *Leu Asp Pro Arg/Ser*, whereby / represents the cleavage site. The high specificity of thrombin towards its substrates and receptors is conferred by its unique anion binding exosite (Goldsack *et al.*, 1998).

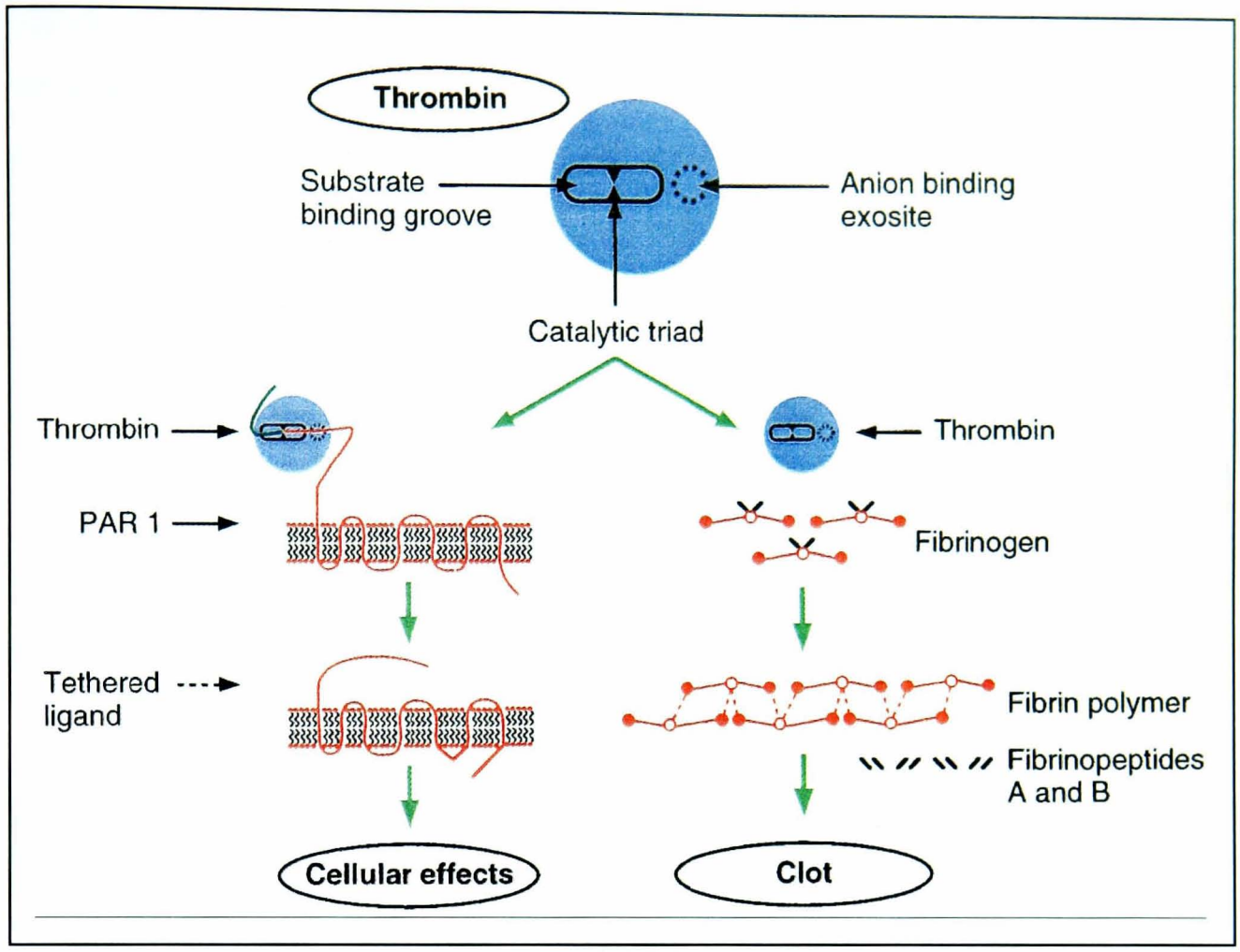

Figure 3.4. Structure and function of thrombin.
A schematic representation of thrombin and its interaction with fibrinogen and PAR-1. Thrombin has several discrete sites; the anion binding exosite of thrombin lies within the deep groove and binds its substrates. The substrate binding sites within this deep groove orientates thrombin's substrates (fibrinogen or PAR-1) enabling cleavage at the catalytic site at an *Arg/Ser* susceptible bond (Goldsack *et al.*, 1998).

Active thrombin mediates the conversion of fibrinogen to fibrin and hence, the formation of the fibrin clot. In addition, thrombin cleaves the FXIIIA subunit releasing an amino terminal activation peptide, resulting in exposure of its active site and initiation of fibrin stabilisation by covalent cross-linking (Wolberg, 2007).

3.1.3.5. Fibrinogen

Fibrinogen is a complex plasma glycoprotein with a molecular weight of 340 KDa (Herrick *et al.*, 1999). Fibrinogen is primarily synthesised in the liver and the plasma concentration is 2-4 g/L (Sidelmann *et al.*, 2000). It consists of two symmetric half molecules consisting of three pairs of disulphide-bonded polypeptide chains termed Aα, Bβ- and γ-chains (*figure 3.5*). Electron microscopy studies of fibrinogen demonstrate a

trinodular structure. The central nodule, termed the E domain represents the amino-terminal disulphide knot of the six chains and the two end nodules termed the D-domains represent the carboxyl terminal regions of the Bβ- and γ-chains and are connected to the E domain by a rod-like α-helical, coiled coil region. The carboxyl terminal is more flexible than the remainder of the fibrinogen molecule and folds back onto the E-domain (Clark, 2003). The three constitutive chains and the two halves of the fibrinogen molecule are held together by a series of 29 disulphide bonds with all 58 cysteine residues of fibrinogen participating in these interactions (Herrick *et al.*, 1999).

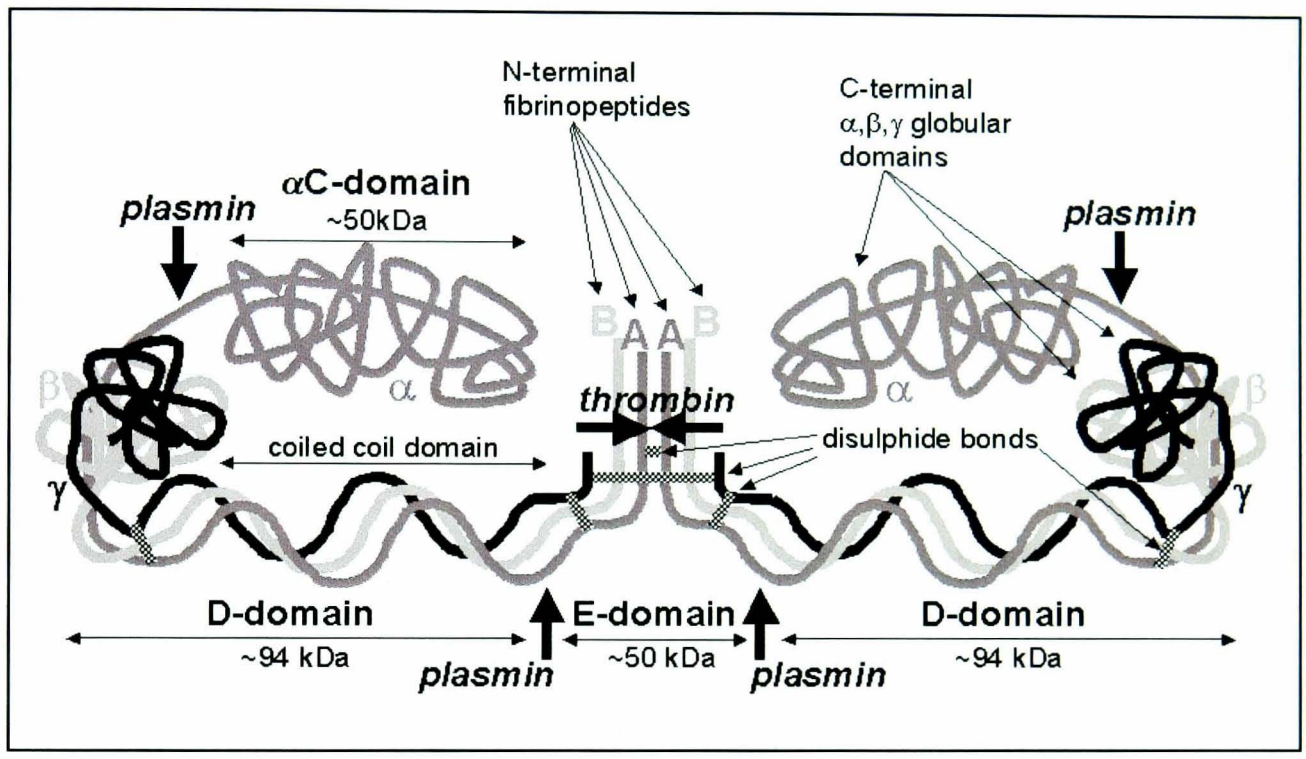

Figure 3.5. Molecular representation of fibrinogen showing the major structural domains. There are three sets of non-identical chains, which are bound by disulphide bonds at the N-terminal end. Removal of FPA and FPB via the action of thrombin leads to cross-linking and formation of the fibrin clot.

The last step in the coagulation cascade involves the conversion of fibrinogen to fibrin by the enzyme thrombin. This is a complex process involving a series of reactions (simplified in *figure 3.6*).

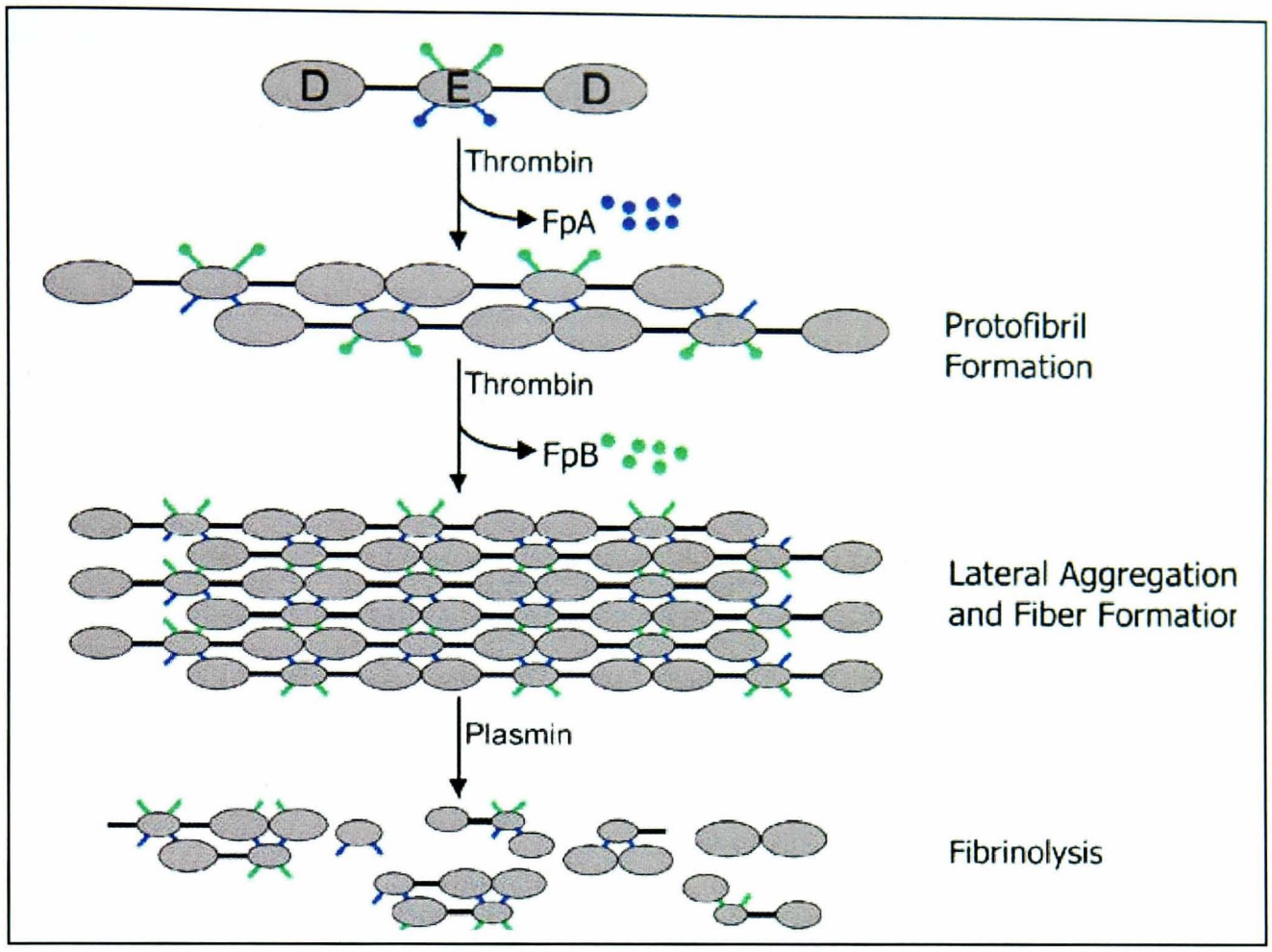

Figure 3.6. A simplified scheme of the thrombin-induced conversion of fibrinogen to fibrin. Thrombin cleaves FPA and FPB from the fibrinogen molecule, converting it to a fibrin monomer that polymerises into soluble fibrin. Simultaneously, thrombin activates FXIII to FXIIIa, which then stabilises the soluble fibrin by generation of irreversible disulphide D-D binding to form cross-linked, insoluble fibrin (Wolberg, 2007).

Thrombin activates fibrinogen by cleaving two small peptides, namely FPA and FPB from the N-terminal regions of the Aα- and Bβ-chains respectively resulting in the exposure of previously cryptic sites, which can then function cooperatively in fibrin polymerisation. Following the release of FPA, new Aα-chain amino terminal ends are generated which interact with sites in the γ-chain carboxyl termini of adjacent molecules to promote non-covalent, lateral assembly. Subsequent removal of FPB creates new Bβ-chain amino-terminal ends that also interact with sites in the γ-chain carboxyl termini of adjacent molecules, reinforcing lateral aggregation and leading to thicker fibrin formation. The activated fibrinogen molecule forms soluble complexes with fibrin and fibrinogen molecules designated fibrin monomers. Due to persistent thrombin activity, the concentration of soluble fibrin monomers increases and subsequently forms long, double-stranded protofibrils. These structures are then combined laterally to form fibrin fibres of varying diameters, resulting in polymerisation of soluble fibrin into covalently

bonded polymerised fibrin. This process is dependent on the presence of thrombin, calcium and activated FXIIIa (Clark, 2003; Mosesson, 1998; Sidelmann *et al.*, 2000).

3.1.3.6. FXIII

Plasma FXIII is a tetrameric molecule composed of two A-subunits of 83.2 KDa and two B-subunits of 79.7 KDa that are bonded non-covalently in a heterologous tetramer of 325.8 KDa (Ashcroft *et al.*, 2000; Schwartz *et al.*, 1973). The A-subunit contains the active site of the enzyme and is synthesised by hepatocytes and monocytes (Nagy *et al.*, 1988). Analysis of the protein phenotype following liver and bone marrow transplantation showed that the A-subunit circulating in the plasma is derived from both the liver and the bone marrow (Wolpl *et al.*, 1987). The B-subunit serves as a carrier for the catalytic A-subunit in plasma, is synthesised by the liver and is secreted as a monomer that binds free A-subunit in plasma (Nagy *et al.*, 1988). The A-subunit is divided into four domains termed; the β-sandwich, the catalytic core, barrel 1 and barrel 2 (Yee *et al.*, 1994). It contains an activation peptide of 37 amino acids that limits the access of the substrate to the active site cysteine. The activation peptide from one subunit of the molecule crosses the opening of the active site on the other. This structure is stabilised by several hydrogen bonds and disulphide bridges between the activation peptide, the β-sandwich and the catalytic core of one subunit and the catalytic core and β-barrel of the second subunit (Weiss *et al.*, 1998). The main function of the B-subunit is the stabilisation and transport of the hydrophobic A-subunit in the aqueous environment of plasma (Ariens *et al.*, 2002).

Plasma FXIII is transformed into an active transglutaminase (FXIIIa) by the proteolytic action of thrombin in the presence of calcium in the final phase of the coagulation cascade. Thrombin removes an activation peptide of 37 amino acid residues from the N-terminal end of subunit-A, then in the presence of calcium, the B-subunits dissociate and subunit-A assumes an enzymatically active configuration. The presence of fibrin greatly accelerates the activation process (Bereczky *et al.*, 2003). In contrast, the cellular form of FXIII is a dimer of the A subunit only (Muszbek *et al.*, 2007).

FXIII plays a vital role in the conversion of the loose meshwork of hydrogen-bonded soluble fibrin into a covalently bonded polymer, which is believed to improve the

mechanical strength, rigidity and elasticity of the clot and increase its resistance to fibrinolysis (Gaffney *et al.*, 1979; Schwartz *et al.*, 1971). FXIIIa also has the capacity to cross-link several plasma proteins to the fibrin clot including plasmin inhibitor, which protects fibrin against plasmin-induced breakdown (Muszbek *et al.*, 1996; Muszbek *et al.*, 1999). The basic mechanism of fibrin cross-linking involves the α- and γ-, but not the β-chains of fibrin. Antiparallel γ-chains of adjacent fibrin monomers are cross-linked quickly by FXIIIa to form γ-dimers. α-Chain cross-linking proceeds more slowly, resulting in the formation of highly crossed α-chain polymers (Sidelmann *et al.*, 2000). The thrombin-mediated cleavage of FPA from fibrinogen and the activation of FXIII occur simultaneously (Brummel *et al.*, 1999), allowing the fibrin clot to be strengthened as it forms.

3.1.4. Vitamin K-dependent carboxylase

Vitamin K-dependent proteins including the coagulation factors (FVII, FIX, FX and prothrombin) and coagulation regulatory proteins (protein C and protein S) require *post*-translational modifications by vitamin K-dependent carboxylase. The enzyme recognises a pro-sequence that precedes the NH2-terminus of the proteins and carboxyl groups are introduced to glutamine residues during protein biosynthesis (Berkner, 2000; Furie *et al.*, 1999). The synthesis of the noted coagulation factors by bronchial epithelial cells would therefore require the presence of vitamin K-dependent carboxylase within the cells. Whole lung extracts have been demonstrated to express a large amount of vitamin-K dependent carboxylase (Houben *et al.*, 1997; Vermeer *et al.*, 1982). Furthermore, alveolar epithelial cells display a significant amount of vitamin K-dependent carboxylase activity (Wallin *et al.*, 1988).

Protein C is a vitamin K-dependent protein that is important in regulating coagulation by forming a complex with thrombomodulin and protein S, which inactivates the coagulation cofactors FV and FVIII (Esmon, 2000). Protein C is produced, *in vitro* by alveolar epithelial cells and has been detected at the gene, protein and activity levels (Hataji *et al.*, 2002). Given that protein C is produced by these cells and that it is capable of being activated indicates that this cell type contains vitamin k-dependent carboxylase and therefore would be capable of introducing the *post*-translation

modifications to vitamin K-dependent proteins. Vitamin K-dependent carboxylase is located within the endoplasmic reticulum; therefore, the processing and secretion of the vitamin K-dependent proteins is likely to occur through a regulated pathway (Furie *et al.*, 1999).

A vitamin K inhibitor, such as warfarin, would dysregulate the carboxylation of vitamin K-dependent proteins (Houben *et al.*, 1997). Warfarin may therefore be a useful tool to investigate whether 16HBE $14o^-$ or NHBE cells demonstrate vitamin K-dependent carboxylase by determining the effects of the inhibitor on the release of vitamin K-dependent coagulation factors.

3.1.5. Inhibition of fibrinogenesis

In addition to the fibrinolytic pathway, whereby the fibrin clot is degraded, the amount of fibrin present at sites of tissue injury is tightly regulated by the actions of inhibitors of the coagulation cascade.

3.1.5.1. Inhibitors of TF:FVIIa

Free FVIIa is unreactive with any of the plasma protease inhibitors; however, when FVII is bound to TF, it becomes susceptible to inhibition. In particular, two plasma inhibitors termed tissue factor pathway inhibitor (TFPI) and antithrombin III can react with the TF:FVIIa complex. TFPI is composed of three Kunitz-type protease inhibitor domains. The first Kunitz domain reacts with the active site of factor VIIa in the TF:FVIIa complex (Broze, 1995). The second Kunitz domain reacts with the active site of FXa. Once the TFPI:Xa complex forms, it binds with higher affinity to TF:FVIIa than does the TFPI molecule alone (Broze, 1995). This results in the formation of a fully inhibited tetramolecular complex. A second molecule related to TFPI, termed TFPI-2 was later identified and shown to inhibit the TF:FVIIa complex (Sprecher *et al.*, 1994). However, the presence of TFPI-2 has not yet been determined in plasma, therefore it is not known whether this molecule plays a physiological role in the regulation of TF-dependent coagulation. Antithrombin III is able to inhibit the activities of all of the serine proteases (FX, thrombin, FIX and FXI), although it is only weakly

inhibitory for the TF:FVIIa complex (Mann *et al.*, 1998). As with TFPI, antithrombin can only react with FVIIa when it is bound to TF. Furthermore, the reaction rate is considerably slow in the absence of heparin (Kondo *et al.*, 1987). Studies have demonstrated that the binding of antithrombin to FVIIa of the TF:FVIIa complex results in the accelerated release of FVIIa, and the resulting FVIIa/antithrombin complex was unable to re-bind to cell surface TF (Rao *et al.*, 1995). Platelet factor 4 (PF4), which can bind to the Gla-domain of vitamin K-dependent FX has also been reported to inhibit TF:FVIIa activation of FX (Slungaard *et al.*, 1994).

3.1.5.2. Inhibitors of thrombin

The proteolytic activity of thrombin is under tight regulatory control. The activity half-life of thrombin in plasma is only 14 seconds as it is rapidly bound by inhibitors, including antithrombin III, α2-macroglobulin, heparin cofactor II, protease nexin-1 and α1-anti-trypsin. The most important mode of inactivation occurs *via* complexing with antithrombin III. This complex is transported to the liver and undergoes degradation in Kupfer cells (Goldsack *et al.*, 1998). In addition to circulating anti-proteases, there are two additional highly efficient thrombin inhibitors, thrombomodulin and Protein S. Thrombin binds to thrombomodulin, leading to the activation of Protein S, inactivating FVa and FVIIIa, preventing further thrombin activation. The binding of thrombin to thrombomodulin also increases the rate at which thrombin is inhibited by plasma inhibitors (Esmon, 2000).

3.1.6. Fibrinolysis

Fibrin provides a matrix for the processes of wound repair, but is not a permanent structure. The fibrinolytic system counterbalances the coagulation system (Astrup, 1952) and when fibrin has performed its duty, it is degraded to soluble fragments. The fibrinolytic process is surface-related and involves a number of circulating (intrinsic) and tissue-related (extrinsic) factors (*figure 3.1*). The fibrin matrix is degraded into a number of fragments, including D-dimers, primarily through the action of plasmin. Plasmin is generated by proteolytic cleavage of the proenzyme plasminogen circulating in human blood. Plasminogen has a high affinity for fibrin and subsequently localises

the fibrinolytic process to the fibrin surface. There are three distinct pathways associated with the activation of plasminogen. Tissue-type plasminogen activator (t-PA), a direct activator of plasminogen, is produced and released from vascular endothelial cells (Pennica *et al.*, 1983). Circulating t-PA is a weak plasminogen activator, but t-PA also binds strongly to fibrin and in consequence, becomes a strong activator of plasminogen (Astrup, 1966). An alternative pathway is related to the intrinsic single-chain urokinase-type plasminogen activator (scu-PA), which can be converted into active urokinase-type plasminogen activator (u-PA) by various proteases (Gunzler *et al.*, 1982). The activation of scu-PA is not known in detail but *in vitro* studies have revealed that plasmin and kallikrein, a serine protease, are responsible for the conversion of scu-PA into u-PA (Binnema *et al.*, 1991; Hauert *et al.*, 1989; Ichinose *et al.*, 1986). The third pathway initiated by activation of coagulation factor XII (FXII) and termed the FXII-dependent pathway. Plasminogen activation is induced by contact activation and FXIIa, kallikrein and FXIa all have the capacity to activate plasminogen directly (Bouma *et al.*, 1978; Mandle *et al.*, 1979; Saito, 1980). Recent studies indicate that assembly of high molecular weight kininogen on its multiprotein receptor allows for prekallikrein activation. On endothelial cells, FXII activation is secondary to prekallikrein activation and amplifies it. The immediate consequence of plasma prekallikrein activation is the cleavage of high molecular weight kininogen with liberation of bradykinin. Bradykinin stimulates t-PA liberation. In addition, formed plasma kallikrein promotes scu-PA activation and subsequent plasminogen activation (Schmaier, 2000). Plasmin degradation of cross-linked fibrin results in the formation of more complex fragments designated as X-oligomers (Gaffney *et al.*, 1979; Graeff *et al.*, 1982). These oligomers consist of the D- and E-fragments of fibrin in various combinations. Large oligomers are released in the initial phase of fibrin breakdown and as the fibrinolytic process continues, the fragments decrease in size. The final fragments generated by degradation of cross-linked fibrin are fragment-E and D-dimer, which consist of two covalently bound D-domains (Doolittle, 1981).

In addition to the plasmin-induced fibrinolytic pathway, other proteases, such as neutrophil elastase, mast cell tryptase, matrix metalloproteinases and cathepsins D and G also have the capacity to degrade fibrin (Herrick *et al.*, 1999). Elastase can facilitate plasmin expression *via* an alternative pathway of plasminogen activation. Elastase modifies plasminogen to yield a zymogen that is a better substrate for activators than

native plasminogen. Furthermore, elastase inactivates plasminogen activator inhibitor-1 (PAI-1) and α_2-antiplasmin without affecting plasmin and plasminogen activators. While plasmin activity develops from a blood zymogen as a consequence of activators synthesized and secreted by endothelium and possibly other cells, elastase is secreted in an active form primarily by polymorphonuclear leukocytes. Plasmin and elastase may play mutual roles in fibrinolysis and inflammation (Machovich *et al.*, 1990).

The potential importance of elastase-mediated fibrin(ogen) degradation was demonstrated in a study involving patients with homozygous plasminogen deficiency. Such patients are not prone to thrombosis and have a normal concentration of fibrinogen in the blood. Fibrinogen and fibrin degradation occur in these patients despite the plasminogen deficiency (Dempfle *et al.*, 1997).

3.1.6.1. Inhibitors of fibrinolysis

There are a number of factors that regulate fibrinolysis. The activity of plasmin and plasminogen activators is regulated by α_2-antiplasmin and PAI-1 respectively (*figure 3*). Moreover, plasminogen activation may be reduced by the binding of other positively charged proteins, such as histidine-rich glycoprotein (Vestergaard *et al.*, 1990) and β_2-glycoprotein I (Schousboe, 1985) to the activating surface. Further regulation of fibrinolysis is achieved *via* the action of thrombin activatable fibrinolysis inhibitor (TAFI). TAFI is activated to TAFIa by thrombin and this reaction is accelerated in the presence of thrombomodulin (Bajzar *et al.*, 1996), a protein in the membrane of endothelial cells. TAFIa removes lysine residues from the fibrin surface and subsequently reduces the amount of plasminogen binding to fibrin. The concentration of thrombin required for the activation of TAFI is greater than that needed for fibrin formation, nevertheless, thrombin has the capacity to activate FXI that in turn, produces more thrombin *via* the intrinsic coagulation cascade (Gailani *et al.*, 1991; Mosnier *et al.*, 1998). Elastase-mediated fibrinolysis is primarily regulated by α_1-protease inhibitor (Bernstein *et al.*, 1994).

3.1.7. Fibrinogen expression *in vitro*

It has previously been shown that the A549 cell line, derived from alveolar basal epithelial cells synthesise their own fibrinogen in response to inflammatory stimuli which is in turn incorporated into a matrix independently of thrombin cleavage (Guadiz *et al.*, 1997; Haidaris, 1997). A549 cells in culture were treated with IL-6 and dexamethasone to induce inflammation and subsequent epithelial injury. In response to injury, northern blot analysis demonstrated that the levels of fibrinogen gamma chain mRNA increased from 2- to 10-fold in the A549 cells. Furthermore, RT-PCR amplification demonstrated increased accumulation of fibrinogen Aα, Bβ and γ chain mRNA in the same cell type. They showed that the newly synthesised fibrinogen was secreted and incorporated into a matrix to support epithelial wound repair.

In view of the evidence that lung alveolar epithelial cells synthesise fibrinogen in response to injury *in vitro*, it was of great interest to investigate the synthesis of coagulation factors by epithelial cells, in the absence of plasma, to form a fibrin matrix to support epithelial repair.

3.1.8. The role of growth factors in bronchial epithelial repair

3.1.8.1. Epidermal growth factor

EGF is a potent mitogen originally isolated by Cohen (1983) from murine salivary glands (Cohen, 1983). Members of the epidermal growth factor family (i.e., EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, and epiregulin) are likely to be important regulators of epithelial restitution by virtue of their ability to stimulate cell migration, proliferation, differentiation, and survival (Kheradmand *et al.*, 1994; Kim *et al.*, 1998; Puddicombe *et al.*, 2000). Immunolocalisation studies have revealed that EGF is primarily expressed in bronchial glands (Aida *et al.*, 1994; Polosa *et al.*, 1999), however, there is weak expression in the bronchial epithelium and smooth muscle (Amishima *et al.*, 1998). The EGF family of growth factors exert their biological effects by binding to and activating EGFR, a 170 kDa tyrosine kinase receptor (Ullrich *et al.*, 1990). EGFR, also known as ErbB1 since it is a member of the ErbB family, is

expressed on the majority of cells types, including fibroblasts and epithelial cells of the bronchial epithelium. The receptors have a restricted expression pattern in polarised lung epithelial cells; with ErbB receptors expressed basolaterally and therefore, are only exposed after epithelial damage. Following epithelial injury, ErbB ligands can activate EGFR or ErbB2 at the edge of the wound, thus facilitating restoration of epithelial integrity (Puddicombe *et al.*, 2000; Vermeer *et al.*, 2003). Expression of EGF and EGFR is upregulated in the epithelium of asthmatic subjects (Puddicombe *et al.*, 2000). More recently, evidence has emerged that while upregulation of EGFR expression is important in repair of the damaged epithelium associated with asthma, impairment of EGFR activity may promote abnormal healing and remodelling, most likely through the uncontrolled release of TGF-β (Knight, 2001). A direct role for EGF in cutaneous wound healing is already well established (Lawrence *et al.*, 1994; McCarthy *et al.*, 1996). In an *in vivo* study involving the gene transfer of a human EGF expression plasmid into wound keratinocytes of porcine skin, Andree *et al* (1994) demonstrated that wounds that were treated with EGF healed 2.1 days quicker compared to controls (Andree *et al.*, 1994). EGF also appears to play a physiologically relevant role in wound healing in the oral epithelium; Noguchi *et al* (1993) demonstrated that EGF promotes healing of superficial tongue wounds in mice. The removal of submandibular salivary glands (the major source of salivary EGF) reduced the rate of wound repair, and the inclusion of EGF in drinking water restored the rate of wound healing to normal levels (Noguchi *et al.*, 1993). Barrow *et al* (1993) showed that administration of aerosolised EGF for 2 weeks enhanced repair of sheep tracheal epithelium after cotton smoke injury *in vivo*. It was suggested that cell proliferation and differentiation were responsible for accelerated epithelial restitution, but the cellular mechanisms were not identified (Barrow *et al.*, 1993). Similarly, in cultured guinea pig airway epithelial cells, EGF was shown to promote the repair of mechanically wounded monolayers by stimulating cell migration with no effect on cell proliferation in the wound margin (Kim *et al.*, 1998). EGF has also been demonstrated to accelerate the repair of mechanical wounds in monolayers of the 16HBE $14o^-$ cell line (Puddicombe *et al.*, 2000). Wadsworth *et al* (2006) demonstrated in an *in vitro* study that 1.0 ng/ml EGF accelerated the rate of wound repair in a monolayer of undifferentiated primary bronchial epithelial cells. This effect was shown to occur *via* an EGFR-dependent mechanism since wound repair was inhibited by a blocking antibody directed to an epitope located in or adjacent to the EGF-binding site of the EGFR. Interestingly, exogenous EGF did not enhance wound

repair of the differentiated, ALI cell model, however, wound repair was nonetheless inhibited by the presence of the same blocking antibody, suggesting that repair of differentiated cell cultures is dependent on autocrine EGF signalling. Repair of the damaged epithelium in undifferentiated cells was shown to occur in the initial stages, predominantly by cell migration and independent of proliferation (Wadsworth *et al.*, 2006). Thus, there is increasing evidence that EGFR is an important factor in the regulation of epithelial repair following injury.

3.1.8.2. Keratinocyte growth factor

Keratinocyte growth factor (KGF) is a 28 kDa peptide classified as a heparin-binding member of the fibroblast growth factor family (FGF-7) (Galiacy *et al.*, 2003). KGF was shown to be produced by various types of mesenchymal cells both *in vitro* and *in vivo* (Smola *et al.*, 1993; Winkles *et al.*, 1997) and to target epithelial cells (Rubin *et al.*, 1989). KGF is recognised as a key growth factor for the bronchial epithelium and has been shown to stimulate proliferation of alveolar epithelial cells *in vitro* (Panos *et al.*, 1993) and *in vivo* (Ulich *et al.*, 1994). Furthermore, *in vivo*, KGF is able to prevent or overcome tissue injury caused by experimental insults (Deterding *et al.*, 1997; Waters *et al.*, 1997; Yi *et al.*, 1996). Using an *in vitro* model of wound repair involving rat alveolar epithelial cells, Galiacy *et al* (2003), observed by video microscopy, that almost immediately after wounding, alveolar epithelial cells form lamellipodia at the wound edge. This was followed by the appearance of plasma membrane protrusions directed toward the wounded area, assembly of fibres and cell migration. The participation of the lamellipodia signified that migration speed was enhanced, since these structures are known to initiate cell mobility. KGF was demonstrated to enhance wound repair at 24 hours compared to the serum-free control; furthermore, wound repair was shown to occur by spreading and migration and not proliferation. Using this *in vitro* model of wound repair, the absence of proliferation is in accordance with other studies (Garat *et al.*, 1996; Kheradmand *et al.*, 1994). The other important observation was that KGF was shown to induce fibronectin expression. Furthermore, KGF enhanced bronchial epithelial cell adhesion to exogenous fibronectin, possibly *via* integrins, in particular, $\alpha5\beta1$ or αv-integrins. Fibronectin is well documented to play a key role in cell migration and tissue repair, therefore by increasing the expression of this molecule; KGF plays an important indirect role in epithelial repair.

3.1.8.3. Transforming growth factor-β

Transforming growth factor (TGF)-β is a multifunctional cytokine with significant regulatory effects on extracellular matrix production from mesenchymal cells in the lung (Coker *et al.*, 1997). More than 30 members of the TGF-β superfamily have been identified and are divided into four main families: TGF-β family, bone morphogenic family, inhibin/activin family and Müllerian-inhibiting substance family (Miyazono *et al.*, 2001). There are three TGF-β isoforms identified in mammals, namely TGF-β1, -β2 and -β3 (Boxall *et al.*, 2006). TGF-β1 and -β2 appear to be the prominent isoforms in the airway (Balzar *et al.*, 2005; Puddicombe *et al.*, 2000; Tschumperlin *et al.*, 2003). Although TGF-β1 was first isolated and characterised in human platelets (Assoian *et al.*, 1983), it is produced in the airways by most cell types, including fibroblasts, smooth muscle, inflammatory and epithelial cells.

TGF-β is produced in an inactive form, tethered to a latency-associated peptide (LAP) by covalent bonding and its signalling through TGF-β receptors requires exposure of the active site of the ligand, either by conformational change or by cleavage of the LAP (Grande, 1997). Large latent TGF-β consists of a mature 25-kDa polypeptide dimer (TGF-β), latency associated protein (LAP), and latent TGF-β binding protein (LTBP). The complex is targeted to the extracellular matrix structures *via* LTBP. Disruption of the non-covalent association between TGF-β and LAP is needed for the release of the active TGF-β, which is then able to bind to the cell surface receptors. Thrombospondin-1 binds to the LSKL sequence in LAP, which leads to conformational changes and TGF-β activation. Integrins, in turn, bind to the RGD recognition sequence in LAP, leading to release of TGF-β either by force directed against the TGF-β complex (αvβ6) or by membrane-type 1 matrix metalloproteinase (MT1-MMP)-dependent proteolytic activity (αvβ8). Reactive oxygen species (ROS) can activate TGF-β directly through oxidation induced conformational change in LAP or indirectly though the activation of proteolytic enzymes (Koli *et al.*, 2008).

Many different cellular responses are elicited by TGF-β, and these are often cell-type specific (Massague, 2000). The complicated signalling patterns triggered by cellular

exposure to TGF-β are incompletely understood. The classic response pathway involves Smad-mediated changes in target gene transcription. The cell surface TGF-β receptor complex is composed of type I and II serine/threonine kinases (Feng *et al.*, 2005). TGF-β binds first to the type II receptor (TGF-βRII), after which the type I receptor (TGF-βRI, ALK-5) is recruited to the complex and activated by phosphorylation. The regulatory Smads 2 and 3 are phosphorylated by TGF-βRI and transported in a complex with Smad4 into the nucleus to alter gene transcription. Specific Smad-binding elements have been identified in the promoter regions of target genes and association with the AP-1 transcription factor has been described in the regulation of gene transcription (Verrecchia *et al.*, 2001).

The three isoforms of TGF-β have been studied extensively with regards to cutaneous wound healing, whereby, TGF-β1 and TGF-β2 have been shown to enhance wound repair (Shah *et al.*, 1995). However, in an *in vitro* study of bronchial epithelial repair, a divergence in function between TGF-β1 and TGF-β2 was identified (Howat *et al.*, 2002). They confirmed that activation mechanisms that facilitate the conversion of latent to active forms of TGF-β are enhanced at an early stage in the repair process and concluded that although active forms of both TGF-β1 and TGF-β2 are produced during wound repair, only TGF-β1 increases the speed of epithelial repair.

TGF-β has also been implicated in the repair phase of airway remodelling (Davies *et al.*, 2003). TGF-β promotes differentiation of fibroblasts into myofibroblasts that secrete interstitial collagens, as well as growth factors, such as endothelin 1 and vascular endothelial growth factor, which are mitogens for smooth muscle and endothelial cells (Richter *et al.*, 2001). It has been demonstrated that human bronchial epithelial cells can produce TGF-β2 at levels that alter gene expression and migration of fibroblasts (Mio *et al.*, 1998; Puddicombe *et al.*, 2000; Tschumperlin *et al.*, 2003). Thus, through the release of TGF-β and its effects on the underlying fibroblasts and myofibroblasts, remodelling signals initiated in the repairing epithelium are propagated and amplified into the deeper layer of the submucosa. Furthermore, TGF-β is reported to prolong the longevity of myofibroblasts by blocking IL-1β–induced apoptosis (Zhang *et al.*, 1999).

Using an *in vitro* model of epithelial-mesenchymal trophic unit (EMTU), referring to the bronchial epithelium and underlying mesenchyme, Thompson *et al* (2006) demonstrated that TGF-β2 regulates tenascin C protein expression and collagen organisation in the matrix under basal conditions and that epithelial injury can increase the concentration of TGF-β2 approximately two-fold and induce changes in the matrix that are consistent with subepithelial fibrosis (Thompson *et al.*, 2006). These findings suggest that regulation of epithelial derived TGF-β2 may be necessary for normal EMTU function.

Dosanjh *et al* (2006) demonstrated in an *in vitro* study, that following inoculation of bronchial epithelial cells with human rhinovirus, expression of TGF-β1 is increased compared to control, both at the level of transcription and translation (Dosanjh, 2006). These findings, along with other studies linking rhinovirus to asthma, suggest that TGF-β has a pathophysiologic role in the development of asthma and remodelling. The pleiotropic cytokine, TGF-β1, has been reported to function as a negative regulator of AHR and airway inflammation in a murine model of asthma (Hansen *et al.*, 2000). TGF-β1 is inhibitory for inflammatory cells such as T cells, B cells, dendritic cells, mast cells and eosinophils, and also modifies the functions of structural cells such as bronchial epithelial cells, fibroblasts and bronchial smooth muscle cells (Hirst, 2000; Holgate, 2000; Wahl, 1992). Interestingly, these cells develop the capacity to produce TGF-β1 following their activation (Wahl, 1992). Thus, it appears that TGF-β1 acts in a negative-feedback loop to suppress activation of these cells. However, persistent activity of TGF-β1 induced by chronic inflammation, which might be caused by repeated stimulation with allergens, could have a detrimental effect and lead to tissue fibrosis or airway remodelling, which results in chronic airflow obstruction. TGF is over-expressed in the asthmatic lung (Boxall *et al.*, 2006). A study of Smad3-knockout mice suggests that there are complexities in understanding the various roles of the TGF-β signalling pathway (Ashcroft *et al.*, 1999), indicating that further investigation will be necessary before the precise role of this cytokine is known. Accumulating knowledge on TGF-β signal transduction will help reveal the detailed mechanisms by which TGF-β1 elicits its effects on target cells in the airways of the experimental models, eventually suppressing AHR and airway inflammation. It is hoped that when the roles of TGF-β1 in human asthma are clearer, the modification of TGF-β1 activity inside or outside the

cells, possibly *via* molecular approaches such as using anti-sense Smad7 oligonucleotide, will offer a potential therapeutic intervention for asthma.

3.1.8.4. Hepatocyte growth factor

Hepatocyte growth factor (HGF) is an 85 kDa growth factor and is synthesised and secreted by mesenchymal cells, such as macrophages, endothelial cells and fibroblasts. HGF is renowned for its ability to exert a number biological functions including mitogenesis, motogenesis, morphogenesis and differentiation in a variety of cell types within the bronchial epithelium (Zahm *et al.*, 2000). Studies by Yanagrita *et al.* (1993), showed evidence of a change in HGF mRNA, HGF activity and HGF *c-met* receptor expression in the rat lung following tracheal epithelial injury, induced by hydrochloride. Furthermore, higher concentrations of HGF were observed in the sera of patients with lung disease compared to that of normal subjects (Yanagita *et al.*, 1993). Studies have demonstrated the presence of elevated levels of HGF in the BAL fluid of patients with pulmonary fibrosis, compared to healthy patients (Sakai *et al.*, 1997). The significance of this finding is that HGF is likely to play a role in the repair of inflammatory lung damage. Little is known about the specific role of HGF in wound repair; however, Zahm *et al* (2003) suggest that the motogenic activity of HGF may be of therapeutic potential in the treatment of bronchial epithelial injury (Zahm *et al.*, 2000).

More recently, Okunishi *et al* (2005) demonstrated a role for HGF in immune regulation (Okunishi *et al.*, 2005). Results of the study revealed that HGF suppressed antigen-induced immune response through inhibition of dendritic cells and thus played a protective role in a murine model of allergic airway inflammation. Antigen presentation from antigen presenting cells (APCs) initiates T cell activation and differentiation. Among APCs, dendritic cells are the most potent and important, thus play a central role and are a logical target in many airway inflammatory diseases involving T cells (Banchereau *et al.*, 1998). Prior to this study, it was emphasized that dendritic cells are crucial in asthma pathogenesis beyond antigen sensitization, and that these cells could be a therapeutic target for asthma (Lambrecht *et al.*, 2003; Lambrecht *et al.*, 1998). Okunishi *et al* (2005) demonstrated that HGF potently suppressed the antigen-presenting capacity of dendritic cells, thus reducing an antigen-induced immune

response. It may therefore be concluded that HGF is of potential benefit in the treatment of asthma (Okunishi *et al.*, 2005).

3.1.9. The role of prostaglandin E_2 in bronchial epithelial repair

Prostaglandin E_2 (PGE_2) is the predominant prostanoid produced by bronchial epithelial cells and is an end product of cyclooxygenase (COX) metabolism of arachidonic acid (Savla *et al.*, 2001).

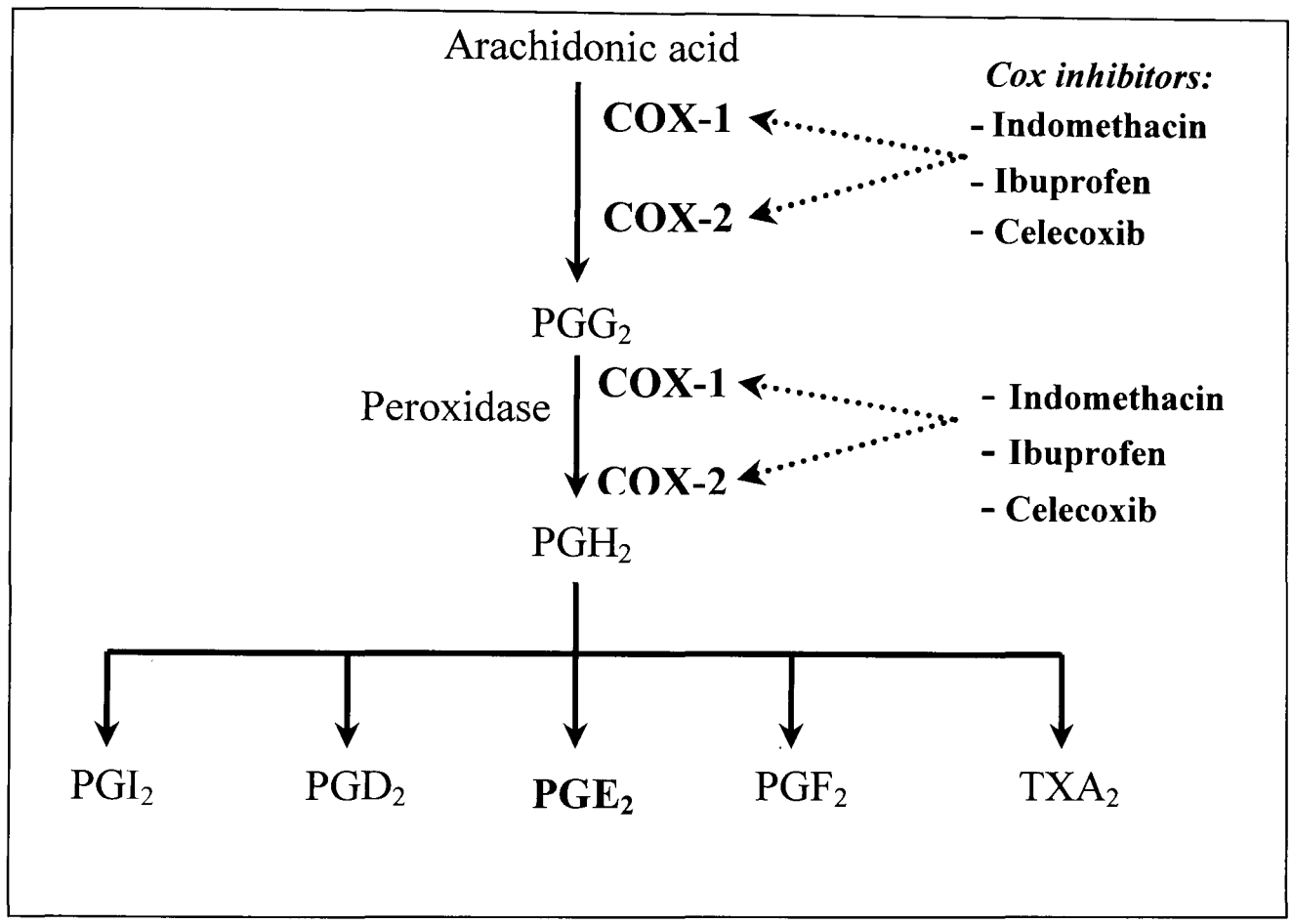

Figure 3.7. The prostaglandin pathway. Bronchial epithelial cells metabolise arachidonic acid to biologically active prostanoids *via* the enzymes cyclooxygenase (COX)-1 and COX-2.

There are two cyclooxygenase enzymes, abbreviated COX-1 and COX-2 that convert arachidonic acid to PGH_2 (Smith *et al.*, 2000). Expression of COX enzymes by epithelial cells is controversial. Mostly, it is believed that COX-1 is constitutively expressed and that COX-2 expression is more tightly regulated and is not expressed under most normal physiological conditions; however, under a variety of pathophysiological conditions, its expression can be rapidly induced, as in asthma (Regan, 2003). However, there are reports that COX-1 is undetectable in A549, 16HBE

14o$^-$ and primary human tracheal epithelial cells in culture; however, COX-2 expression was found to be constitutive in both stimulated and unstimulated cells (Asano *et al.*, 1996; Walenga *et al.*, 1996). Arachidonic acid is oxidised to PGG_2 *via* COX activity, which in turn is reduced to PGH_2. PGH_2 is then converted to the final prostanoid products through the actions of specific synthases; in particular, PGE_2 synthase catalyses the conversion of PGH_2 to PGE_2 (*figure 3.7*) (Kamei *et al.*, 2003). In addition to bronchial epithelial cells, PGE_2 is produced by a variety of cell types in the airway, including smooth muscle, alveolar macrophages and pulmonary endothelial cells (Meyrick *et al.*, 1989; Widdicombe *et al.*, 1989).

PGE_2 has been shown to exert a variety of anti-inflammatory and bronchoprotective effects both *in vitro* and *in vivo*. PGE_2 may prevent allergen-induced bronchoconstriction (Gauvreau *et al.*, 1999), relax airway smooth muscle (Knight *et al.*, 1995), inhibit cholinergic neurotransmission (Ito *et al.*, 1990) and modulate fibroblast proliferation (McAnulty *et al.*, 1997). PGE_2 mediates its effects *via* G protein-coupled receptors to alter cyclic nucleotide levels in cells or stimulate phosphatidylinositol, thereby causing effects such as airway smooth muscle relaxation or uterine muscle contraction (Sheller *et al.*, 2000). The receptors for PGE_2 have been classified into four subtypes, entitled E-prostanoid (EP)-1, EP-2, EP-3 and EP-4. These EP receptors modulate a variety of effects: EP-1 receptors binding PGE_2 signal *via* G proteins to activate phospholipase C, generate phosphatidylinositol, mobilise calcium and create a chloride current (Breyer *et al.*, 1998); EP-2 and EP-4 receptors characteristically relax smooth muscle by signalling through a G_s to increase intracellular cyclic adenosine monophoshate (cAMP) levels and the EP_3 receptor consists of a number of splice variants displaying various degrees of constitutive activity (Jin *et al.*, 1997).

In vitro studies have demonstrated that PGE_2 causes relaxation of airway smooth muscle and also has effects on airway cholinergic nerve activity (Madison *et al.*, 1989; Sweatman *et al.*, 1968). Furthermore, PGE_2 released from epithelial cells has been shown to inhibit vagal cholinergic efferent transmission *via* a prejunctional mechanism (Matsumoto *et al.*, 1996). Fortner *et al.* (2001) reported that relaxation of constricted mouse tracheal smooth muscle by substance P and ATP is mediated through PGE_2 acting on EP-2 receptors (Fortner *et al.*, 2001). A number of *in vivo* studies involving inhalation of PGE_2 by human subjects have revealed that the prostanoid may cause

airway constriction or bronchodilation, which may be dependent on the timing of measurements following PGE_2 administration (Mathe *et al.*, 1975; Walters *et al.*, 1982). Sheller *et al* (2000) demonstrated that bronchodilation and thus relaxation of airway smooth muscle results from the activation of EP_2 receptors in a murine model (Sheller *et al.*, 2000). Conversely, PGE_2-mediated airway constriction is dependent on activation of the EP-1 and EP-3 receptors (Tilley *et al.*, 2003). In the cat trachea, PGE_2 acts as a bronchodilator but is a constrictor in the guinea pig ileum (Gardiner, 1986). Clinical studies have reported that, for the majority of asthmatic patients, PGE_2 produces beneficial bronchodilatory and bronchoprotective effects, however, a subset of patients have developed profound bronchoconstriction in response to PGE_2 (Cuthbert, 1969; Mathe *et al.*, 1975), hence the reason that PGE_2 has never been exploited as a therapy for asthma. Ideally, the development of a suitable EP_2 receptor-specific agonist to generate a bronchodilatory response would be a potential treatment for asthma.

In vitro studies by Savla *et al* (2001) showed that inhibition of COX with indomethacin resulted in a dose-dependent inhibition of wound repair in both human and cat species of bronchial epithelial cells. Specific inhibitors of both COX-1 and COX-2 isoforms, resulted in impairment of wound repair. This effect was overcome by the addition of exogenous PGE_2, which was shown to enhance the rate of wound repair in a dose-dependant manner. Importantly, inhibition of COX with indomethacin only resulted in sustained inhibition of wound repair at initial time-points, suggesting that this prostanoid is involved in the early repair process of spreading and migration. The fact that indomethacin inhibits wound repair signifies that PGE_2 is secreted by bronchial epithelial cells to promote wound repair and it is likely that this effect is mediated *via* the EP-1 and EP-4 receptor subtypes (Savla *et al.*, 2001).

3.1.10. IL-8

IL-8 is an inflammatory chemokine that is abundantly expressed in the bronchial epithelium. IL-8 is considered a potent chemoattractant for neutrophils (Kunkel *et al.*, 1991) and eosinophils (Shute, 1994; Walker *et al.*, 1994). There is increasing evidence to suggest that IL-8 is a marker of severe asthma. The presence of IL-8 was demonstrated in the sera and bronchial tissue of subjects with severe atopic asthma, that was undetectable in that of normal subjects and subjects with mild atopic asthma (Shute

et al., 1997). More recently, Silvestri *et al.* (2006) confirmed that severe asthmatics demonstrate higher concentrations of IL-8 in serum compared to mild-moderate asthmatics or healthy controls (Silvestri *et al.*, 2006). Furthermore, it was recently shown that epithelial expression of the neutrophil chemoattractant, IL-8 is increased in severe asthma and its appearance correlates with increased EGFR expression as a marker of epithelial damage. Further *in vitro* studies have revealed the EGF treatment of primary bronchial epithelial cells enhances IL-8 release (Hamilton *et al.*, 2003). Since the EGFR is over-expressed in the epithelium of patients with severe asthma and strongly correlates with IL-8 expression, this may contribute to airway neutrophilia in patients with severe asthma. It has been suggested that TGF-α is the ligand for the EGFR mediated IL-8 release from bronchial epithelial cells in response to cigarette smoke (Richter *et al.*, 2002).

3.1.11. Neutrophil elastase

Neutrophil elastase, a member of the chymotrypsin superfamily of serine proteases, is a 33 kDa enzyme with several isoforms that differ in their extent of glycosylation (Bruch *et al.*, 1986; Ohlsson *et al.*, 1974). In neutrophils, the concentration of neutrophil elastase exceeds 5 mM and its total cellular amount has been estimated to be up to 3 pg. Such a high concentration of elastase is tightly regulated by compartmentalisation in the azurophil granules (Bruch *et al.*, 1986). Upon activation, neutrophil elastase is rapidly released from the granules into the extracellular space with a portion remaining bound to the neutrophil plasma membrane (Owen *et al.*, 1995) and its activity is regulated by multiple endogenous inhibitors. It has also been reported that neutrophils contain α_1-protease inhibitor (du Bois *et al.*, 1991).

Neutrophil elastase has been demonstrated to play a role in stimulating mucus secretion (Fahy *et al.*, 1992), decreasing ciliary function (Amitani *et al.*, 1991), increasing epithelial permeability (Peterson *et al.*, 1995) and tissue destruction (Janoff, 1985); and in doing so, may contribute to several inflammatory disorders, including emphysema (Snider *et al.*, 1991), chronic bronchitis (Weitz *et al.*, 1987), bronchiectasis (Stockley *et al.*, 1984), cystic fibrosis (Jackson *et al.*, 1984), adult respiratory distress syndrome (ARDS) (Lee *et al.*, 1981) and there is accumulating evidence to suggest that neutrophil elastase is associated with severe asthma (Fahy *et al.*, 1995; Holgate *et al.*, 2006;

Vignola *et al.*, 1998; Wenzel *et al.*, 1999; Wenzel *et al.*, 1997). However, human airway epithelial cells (HAEC) are reported to play important bronchoprotective and immunomodulatory roles in chronic neutrophilic inflammation. A study involving the stimulation of HAEC with neutrophil elastase demonstrated a significant increase in PGE_2 release *via* activation of p44/42 MAP kinases and upregulation of COX-2 (Perng *et al.*, 2003). Neutrophil elastase is considered one of the most destructive enzymes due to its ability to degrade almost all extracellular matrix proteins (Chua *et al.*, 2006) and soluble proteins including coagulation factors V, VII, X, VIII, XI and XIII (Havemann *et al.*, 1984; Okada *et al.*, 1988), leading to their loss of function. See *table 3.1* for a more specific list of the proteolytic substrates of neutrophil elastase. In addition to its proteolytic activity, neutrophil elastase is also known to induce the expression of IL-6, IL-8, GM-CSF (Bedard *et al.*, 1993) (Nakamura *et al.*, 1992) and mucin from epithelial cells (Sommerhoff *et al.*, 1990), and the release of TGF-β which binds to extracellular matrix (Taipale *et al.*, 1995). Interestingly, neutrophil elastase degraded fragments such as those from fibrin (Cepinskas *et al.*, 1997) and laminin (Steadman *et al.*, 1993) are known to be chemotactic toward neutrophils.

ECM components	**Soluble proteins**
Elastin and elastic fibres	Coagulation factors
Collagen (types II, III, IV, VI, VIII, IX and X)	Complement factors
Fibrillin microfibrils	Fibrinogen
Fibrin (cross-linked)	Immunoglobulins
Fibronectin	Proenzymes
Laminin	Tenascin
Proteoglycans	TNF-α
Latent TGF-β1	Proteinase inhibitors
Bubepidermal BP-180	MMPs
	TIMP
	Surfactant protein D

Table 3.1. The proteolytic substrates of neutrophil elastase.

Until relatively recently, all asthma cases were regarded as being similar, differing only in severity and therefore treatment type; but research over the last two decades has identified subphenotypes of asthma that differ in their allergic pathways. Most mild-moderate asthma is associated with atopy, but the most severe and chronic phenotype of asthma is now commonly associated with greater involvement of neutrophils and evidence of tissue destruction and airway remodelling. With regards to wound repair,

appropriate regulation of neutrophil elastase in the normal airway can be beneficial, particularly in the degradation and removal of the fibrin matrix, once it has performed its duty. However, the excessive abundance of this enzyme may contribute to the chronic inflammation and remodelling associated with severe asthma.

3.2. Aims and objectives

The predominant aim of this section was to determine the expression of coagulation factors by 16HBE 14o$^-$ cells cultured in serum free medium at the protein level using immunoblot and immunohistochemistry. Further aims were to investigate the contribution of growth factors by analysis of their concentration in cell culture supernatants derived from 16HBE 14o$^-$ cells by ELISA. Finally, it was of interest to investigate the contribution of inflammatory mediators including PGE2, IL-8 and neutrophil elastase by analysis of their concentration in cell culture supernatants from 16HBE 14o$^-$ cells using immunoblot and ELISA assays.

3.3. Methods

3.3.1. Immunostaining for TF

16HBE 14o$^-$ cells were cultured in Microtek 8-well chamber slides (Nunc) at 50,000 cells per well for 48 hours. At confluence, cells were washed with 500 µl of 1X PBS per well and quiesced for 16 hours in MEM-ITS. Prior to each experiment, MEM-ITS was refreshed. Cells were mechanically wounded with a P2 Gilson pipette tip in a cross-hatch manner (+) and returned to the incubator at 37°C for a time-course of 30 minutes, 1 hour and 2 hours. Supernatants were removed and cells were washed with 500 µl of 1X PBS per well. Cells were fixed by adding 500 µl per well of 4% ($^w/_v$) paraformaldehyde in PBS for 30 minutes at room temperature then washed 3 times by adding 500 µl of 1 X PBS per well and permeabilised by adding 500 µl per well of ice-cold methanol (100%), for 5 minutes at -20°C. Cells were washed 3 times by adding 500 µl of 1 X PBS per well and incubated for 16 hours in PBS containing 1% ($^w/_v$) bovine serum albumin (BSA) at 4°C to block non-specific binding sites. Cells were

incubated with primary antibody (mouse anti-human TF, 1 mg/ml, American Diagnostica, Dundee, UK) diluted 1 in 200 in 1X PBS-1% BSA for 1 hour at room temperature. Cells were washed 5 times for 5 minutes each with 500 µl of 1X PBS-0.02% ($^{v}/_{v}$) Tween-20 per well and incubated with secondary antibody (goat anti-mouse Alexa Fluor® 488 $F(ab')_2$ fragment, Molecular Probes, UK), diluted 1 in 400 in 1X PBS-1% BSA for 1 hour at room temperature. Cells were washed 5 times for 5 minutes each with 500 µl of 1X PBS-0.02% ($^{v}/_{v}$) Tween-20 and the slide mounted using Vectashield mounting medium (Vector Labs, UK). Cells were viewed using a Zeiss LSM 510 confocal microscope.

3.3.2. TF activity assay

The TF activity assay was conducted on cells in a 96-well plate, using an adaptation of a method (Lwaleed *et al.*, 2001). 16HBE 14o^{-} cells were seeded at a density of 25,000 cells per well in a 96-well plate and grown to confluence for 2 days. At confluence, the cells were washed in 1X PBS and quiesced overnight in serum-free MEM-ITS. Immediately prior to experiment, the MEM-ITS was discarded, cells were washed in 1X PBS and 90 µl fresh MEM-ITS was added to each well. Cells were mechanically wounded in a cross-hatch manner (+) and incubated at 37 °C for 2 hours. Following incubation the supernatants were removed and the cells washed in 1X PBS. The activity assay was conducted to determine the cell surface TF activity in response to wounding. To each well, 60 µl of 0.05 M Tris-NaCl pH 7.8, 20 µl of 0.025 M $CaCl_2$ and 15 µl of 0.75 IU/ml Prothromplex TIM-4 were added to the 96-well plate which was then incubated for 15 minutes at 37°C. Prothromplex TIM-4 is a concentrate of the human coagulation factors II, VII, IX and X that can collectively form the prothrombinase complex. The cascade is initiated through the interaction of FVII with TF and the activity of FX was measured, as this was proportional to the TF activity of the cells. After the 15 minute incubation period the further generation of coagulation factors by the extrinsic coagulation pathway was prevented by adding 100 µl 0.05 M Tris-NaCl pH 7.8 containing 7 mM EDTA. The activity of FX was measured by the addition of 40 µl 1 mM S-2222. The plate was read at 405 nm (Dynex MRX microplate reader. West Sussex, UK) for 1 hour at 37°C.

3.3.3. Immunoblotting

16HBE 14o$^-$ cells were cultured for 48 hours in 24-well plates until fully confluent and quiesced for 16 hours in 1 ml per well of serum-free MEM-ITS, as described in *section 2.2.1.* Prior to wounding, MEM-ITS was refreshed, a concentration range of drugs were added to the cells and the plates were incubated for 30 minutes at 37°C. Cells were mechanically wounded with a P2 Gilson pipette tip in a cross-hatch manner with different degrees of wounding, as described in *section 2.2.5*, and returned to the incubator at 37°C for 20 minutes and 2 hours. Supernatants were removed from cells and centrifuged for 7 minutes at 670 x *g* (ALC PK120 Centrifuge, Winchester Virginia, USA) to remove cell debris.

Fibrinogen and FXIII

For the purpose of immunoblotting, 3 µl of supernatant from each well was applied to 0.45 µm nitrocellulose membrane, using an 8 x 12 grid to locate the spots. A series of plasma standards in the range of 0.01-10% were prepared from normal plasma (Alpha Laboratories, Hampshire, UK), diluted in MEM-ITS and added to the membrane as a reference. The membrane was left to dry at room temperature for 60 minutes and incubated for 16 hours at 4°C in 1X PBS-2% ($^v/_v$) Tween-20 to block non-specific binding sites. Membrane blots were then washed three times for 10 minutes each in PBS-0.05% ($^v/_v$) Tween-20, with shaking at room temperature.

For the detection of fibrinogen (sheep anti-human FPA IgG-peroxidase conjugate, 2 mg/ml, Affinity Biologicals) and FXIII (sheep anti-human FXIIIA IgG-peroxidase conjugate, 2 mg/ml, Affinity Biologicals), antibodies were diluted in PBS-2% ($^v/_v$) Tween-20 (FXIII: 1 in 20,000 and fibrinogen: 1 in 10,000) and 5 ml of the antibody solution was applied to each membrane blot. Membrane blots were placed on parafilm in plastic trays and incubated with antibodies for 60 minutes at room temperature. Membrane blots were washed five times for 10 minutes each in PBS-0.05% ($^v/_v$) Tween-20 with shaking at room temperature.

D-dimers

For the purpose of immunoblotting, 20 µl of supernatant and standard was applied to 0.45 µm nitrocellulose membrane using a dot blot hybridisation manifold (Jencons PLS, UK) under vacuum. A concentration range of D-dimer standards were prepared using a high molecular weight D-dimer standard (American Diagnostica: 492 ng/ml). The nitrocellulose membranes were left to dry at room temperature for 1 hour and incubated for 16 hours at 4°C in 1X PBS-2% ($^{v}/_{v}$) Tween-20 to block non-specific binding sites. Membrane blots were washed three times for 10 minutes each in PBS-0.05% ($^{v}/_{v}$) Tween-20, with shaking at room temperature.

For the detection of D-dimers, the primary antibody (mouse anti-D-dimer, 0.5 mg/ml, American Diagnostica) was diluted 1 in 500 in 1X PBS-2% ($^{v}/_{v}$) Tween-20. To each membrane blot, 5 ml of the antibody solution (1 µg/ml) was applied. Membrane blots were placed on parafilm in plastic trays and incubated with antibody for 1 hour at room temperature. Blots were washed three times for 10 minutes each in PBS-0.05% ($^{v}/_{v}$) Tween-20, with shaking at room temperature. Secondary antibody (biotinylated goat anti-mouse, Dako) was diluted 1 in 10,000 in 1X PBS-2% ($^{v}/_{v}$) Tween-20 and 5 ml of the antibody solution was applied to each membrane blot and incubated for 1 hour at room temperature. Blots were washed three times for 10 minutes each in PBS-0.05% ($^{v}/_{v}$) Tween-20, with shaking. Membranes were incubated in 40 ml SAB complex (StreptABComplex) for 45 minutes at room temperature. The SAB complex was prepared 90 minutes prior to use by adding 2 µl A (streptavidin) and 2 µl B (biotin-HRP) to 496 µl PBS and made up to 40 ml with PBS-2% ($^{v}/_{v}$) Tween-20 immediately before use. The blots were washed five times for 10 minutes each with PBS-0.05% ($^{v}/_{v}$) Tween-20, with shaking at room temperature.

For chemiluminescent detection following immunoblotting for fibrinogen, FXIII and D-dimers, Pierce West Pico SuperSignal substrate (Perbio, Northumberland, UK) was used. Activator and peroxidase solutions were added together in a 1:1 ratio and 5 ml was added to the membrane blot for 5 minutes at room temperature. Blots were transferred into plastic, zip-lock bags and excess substrate removed by blotting. Blots were placed in a cassette and exposed to X-ray films overnight. Films were developed

using the Kodak X-ray developer and the intensity of the dots were measured using ChemiGenius GeneSnap densitometry software.

3.3.4. Lactate dehydrogenase (LDH) assay

The lactate dehydrogenase (LDH) assay (TOX-7, Sigma, Dorset, UK) was used to determine whether the effects of wounding on the release of coagulation factors were due to cytotoxicity. LDH is an intracellular cytoplasmic protein that is released into cell culture supernatants when cells become non-viable and sustain damage to their membrane (Legrand *et al.*, 1992). The assay works on the principle that LDH released from cells, reduces NAD to NADH, and the NADH then converts a tetrazolium dye into a soluble, coloured formazan derivative. The assay was adapted for use in a 96-well plate. Cell culture supernatants and cell lysates were diluted 1 in 10 by adding 5 µl of sample to 45 µl of 1X PBS. The LDH assay solution was prepared by mixing equal volumes of LDH substrate, LDH enzyme and LDH tetrazolium dye. To each well, 25 µl of the LDH assay mixture was added. The plate incubated for 5 minutes at room temperature, protected from light. In order to stop the reaction, 8.33 µl of 1M HCl was added to each well and the absorbance at 490 nm was measured using a microplate reader (Dynex MRX microplate reader, West Sussex, UK).

3.3.5. Immunoassays for growth factors

16HBE 14o$^-$ cells were seeded at a density of 400,000 cells per well in 6-well plates and cultured for 48 hours in 2 ml per well of full MEM until fully confluent. At confluence, cells were quiesced for 16 hours in 1 ml per well of serum-free MEM-ITS. Prior to experiment, MEM-ITS was refreshed. Cells were maximally wounded, i.e. 4 horizontal scrapes and 4 vertical scrapes, as described in *section 2.2.5* and subsequently incubated for 2, 4, 6, 8 and 10 hours at 37°C. The supernatants were removed from cells and centrifuged for 7 minutes at 670 x *g* (2000 rpm: ALC PK120 Centrifuge, Winchester Virginia, USA) to remove cell debris.

Human EGF

Centrifuged supernatants were analysed for EGF using a Quantikine® human EGF immunoassay (R&D Systems). EGF standards were prepared in 1X calibrator diluent RD5E (provided) in the concentration range of: 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 pg/ml. To each well, 200 µl of standards and supernatants were added and the plate was covered and incubated for 2 hours at room temperature. Wells were aspirated and washed by adding 400 µl of wash buffer (provided) to each well. This was repeated for a total of three washes, ensuring complete removal of wash buffer each time. After the final wash, wells were aspirated and any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. To each well, 200 µl of EGF conjugate was added and the plate was covered and incubated for 1 hour at room temperature. Wells were aspirated and washed for a total of three washes, as previously described. Substrate solution was prepared 15 minutes before required by adding equal volumes of colour reagents A and B together and protecting from light. 200 µl of the resultant substrate solution was added to each well, the plate was covered and incubated for 20 minutes at room temperature, protected from light. In order to stop the reaction, 50 µl of stop solution (2 N sulphuric acid) was added to each well.

Human KGF

Centrifuged supernatants were analysed for KGF using a Quantikine® human KGF immunoassay (R&D Systems). To each well, 100 µl of assay diluent RD1-25 was added. KGF standards were prepared in calibrator diluent RD5R (provided) in the concentration range of 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/ml. To each well, 100 µl of standards and cell culture supernatants was added and the plate was covered and incubated for 3 hours at room temperature. Wells were aspirated and washed by adding 400 µl of diluted wash buffer (provided) to each well for a total of three washes, ensuring complete removal of liquid each time. After the final wash, wells were aspirated and any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. To each well, 200 µl of KGF conjugate was added and the plate was covered and incubated for 1.75 hours at room temperature. Wells were aspirated and washed as previously described. Substrate solution was

prepared 15 minutes before required by adding equal volumes of colour reagents A and B together and protecting from light. To each well, 200 µl of the substrate solution was added and the plate was covered and incubated for 30 minutes at room temperature, protected from light. In order to stop the reaction, 50 µl of stop solution (2 N sulphuric acid) was added to each well.

Human TGF-β1

Centrifuged supernatants were analysed for TGF-β1 using a Quantikine® human TGF-β1 immunoassay (R&D Systems). TGF was assayed as active and total TGF-β1, before and after acid activation of the samples respectively. To acid activate TGF-β1, the following procedure was carried out in a separate 96-well plate and samples were assayed after neutralisation (pH 7.2-7.6). To 100 µl of cell culture supernatant in the well, 20 µl of 1 N HCL was added and the plate was incubated for 10 minutes at room temperature. The acidified sample was neutralised by adding 20 µl of 1.2 N NaOH/0.5 M HEPES. TGF-β1 standards were prepared in calibrator diluent RD5-26 (provided) in the concentration range of 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/ml. To each well of the 96-well plate provided, 50 µl of assay diluent RD1-21 (provided) was added. This was followed by the addition of 50 µl of TGF-β1 standard and samples containing active and total TGF-β1 to each well. The plate was tapped gently to mix, covered and incubated for 2 hours at room temperature. Wells were aspirated and washed by adding 400 µl of wash buffer to each well for a total of three washes, ensuring complete removal of liquid each time. After the final wash, wells were aspirated and any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. To each well, 100 µl of TGF-β1 conjugate was added and the plate was covered and incubated for 2 hours at room temperature. The wells were aspirated and washed as previously described. Substrate solution was prepared 15 minutes before required by adding equal volumes of colour reagents A and B together and protecting from light. To each well, 100 µl of the substrate solution was added and the plate was covered and incubated for 30 minutes at room temperature, protected from light. In order to stop the reaction, 100 µl of diluted HCL (provided) was added to each well.

Human HGF

Centrifuged supernatants were analysed for HGF using a Quantikine® human HGF immunoassay (R&D Systems). To each well of a 96-well plate pre-coated with a murine monoclonal antibody against HGF, 150 µl of assay diluent RD1W was added. HGF standards were prepared in 1X calibrator diluent RD5P (provided) in the concentration range of 125, 250, 500, 1000, 2000, 4000 and 8000 pg/ml. To each well, 50 µl of standards and cell culture supernatants was added and the plate was covered and incubated for 2 hours at room temperature. Wells were aspirated and washed by adding 400 µl of diluted wash buffer (provided) to each well for a total of three washes, ensuring complete removal of liquid each time. After the final wash, wells were aspirated and any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. To each well, 200 µl of HGF conjugate was added and the plate was covered and incubated for 1.75 hours at room temperature. Wells were aspirated and washed as previously described. Substrate solution was prepared 15 minutes before required by adding equal volumes of colour reagents A and B together and protecting from light. To each well, 200 µl of the substrate solution was added and the plate was covered and incubated for 30 minutes at room temperature, protected from light. In order to stop the reaction, 50 µl of stop solution (2 N sulphuric acid) was added to each well.

For each growth factor assay, the optical density of each well was determined using a microplate reader (Dynex MRX: West Sussex, UK) set to 450 nm. The optical density at 540 nm was also determined and subtracted from the optical density at 450 nm to correct for any optical imperfections in the plate.

3.3.6. Immunostaining for HGF, *c-Met* receptor and FXIII

16HBE 14o$^-$ cells were cultured in Microtek 8-well chamber slides (Nunc) at 50,000 cells per well for 48 hours. At confluence, cells were washed with 500 µl of 1X PBS per well and quiesced for 16 hours in MEM-ITS. Prior to each experiment, MEM-ITS was refreshed. A final concentration of 100 ng/ml HGF (Sigma, Dorset, UK) was added to cells, which were then incubated at 37°C for 30 minutes. Cells were mechanically

wounded with a P2 Gilson pipette tip in a cross-hatch manner (+) and returned to the incubator at 37°C for a time-course of 2 hours. Supernatants were removed and cells were washed with 500 µl of 1X PBS per well. Cells were fixed by adding 500 µl per well of 4% ($^{w}/_{v}$) paraformaldehyde in PBS for 30 minutes at room temperature then washed 3 times by adding 500 µl of 1 X PBS per well and permeabilised by adding 500 µl per well of ice-cold methanol (100%), for 5 minutes at -20°C. Cells were washed 3 times by adding 500 µl of 1 X PBS per well and incubated for 16 hours in PBS containing 1% ($^{w}/_{v}$) BSA at 4°C to block non-specific binding sites. Cells were incubated with primary antibodies: mouse anti-human HGF (0.2 mg/ml stock diluted in sterile 1X PBS; R&D Systems, Oxfordshire, UK), goat anti-human c-Met (0.1 mg/ml stock diluted in sterile 1X PBS; R&D Systems, Oxfordshire, UK), and sheep anti-human FXIII (2 mg/ml stock diluted in DMSO; Affinity Biologicals, distributed by Quadratech Diagnostic Ltd, Surrey, UK), diluted 1 in 400 in 1X PBS-1% BSA for 1 hour at room temperature. Cells were washed 5 times for 5 minutes each with 500 µl of 1X PBS-0.02% ($^{v}/_{v}$) Tween-20 per well and incubated with secondary antibodies for c-Met receptor and FXIII (Donkey anti-sheep Alexa Fluor® 594, 2 mg/ml; Molecular Probes, UK) and HGF (goat anti-mouse Alexa Fluor® 488 $F(ab')_2$ fragment, 2 mg/ml; Molecular Probes, UK), diluted 1 in 400 in 1X PBS-1% BSA for 1 hour at room temperature. Cells were washed 5 times for 5 minutes each with 500 µl of 1X PBS-0.02% ($^{v}/_{v}$) Tween-20 and the slide mounted using Vectashield mounting medium (Vector Labs, UK). Cells were viewed using a Zeiss LSM 510 confocal microscope.

3.3.7. Immunoassay for PGE_2

16HBE 14o^{-} cells were cultured for 48 hours on 24-well plates until fully confluent and quiesced for 16 hours in serum-free MEM-ITS, as described in *section 2.2.1.* Following mechanical wounding and subsequent incubation for 20 minutes and 2 hours, the supernatants were removed from cells and centrifuged for 7 minutes at 670 x *g* (2000 rpm: ALC PK120 Centrifuge, Winchester Virginia, USA) to remove cell debris then analysed for PGE_2 using a Parameter™ PGE_2 high sensitivity immunoassay (R&D Systems, Oxfordshire, UK). This assay is based on the competitive binding technique in which PGE_2 present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labelled PGE_2 for sites on a mouse monoclonal antibody. During the

incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash step to remove the excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The colour development is stopped, and the absorbance is read at 450 nm. The intensity of the colour is inversely proportional to the concentration of PGE_2 in the sample.

PGE_2 standards were prepared in calibrator diluent RD5-39 in the concentration range of: 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/ml. Calibrator diluent RD5-39 was added to non-specific binding (NSB) wells (150 µl) and zero standard B_0 wells (100 µl) and 100 µl of standards and supernatants were added to remaining wells. To each well, 50 µl of primary mouse monoclonal antibody to PGE_2 was added, excluding NSB wells, followed by the addition of 50 µl of PGE_2 conjugate to each well. The plate was covered and incubated for 20 hours at 4°C. Wells were aspirated and washed by adding 400 µl of diluted wash buffer (provided) for a total of four washes, ensuring complete removal of liquid after each wash. After the final wash, wells were aspirated and any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Substrate solution was prepared 15 minutes before required by mixing together equal volumes of colour reagents A and B and protecting from light. To each well, 200 µl of the substrate solution was added and the plate was incubated for 20 minutes at room temperature, protected from light. In order to stop the reaction, 50 µl of stop solution (2 N sulphuric acid) was added to each well and the optical density of each well was determined using a microplate reader (Dynex MRX: West Sussex, UK) set to 450 nm. The optical density at 540 nm was also determined and subtracted from the optical density at 450 nm to correct for any optical imperfections in the plate.

3.3.8. Immunoassay for IL-8

16HBE $14o^-$ cells were cultured, wounded and incubated and supernatants were harvested as described in *section 3.3.5*. Centrifuged supernatants were analysed for IL-8 using a Pelikine Compact™ human IL-8 ELISA kit (Sanquin Reagents, Amsterdam). Monoclonal anti-human IL-8 antibody was diluted 1 in 100 in coating buffer (coating buffer: 1 capsule of carbonate/bicarbonate (Sigma, UK) dissolved in 100 ml deionised

H_2O). 100 µl of coating antibody was added to each well and the plate was covered and incubated overnight at room temperature. Prior to use, all reagents (with the exception of streptavidin-HRP) were brought to room temperature. The plate was washed 5 times with 200 µl per well of 1X PBS. To each well, 200 µl of blocking buffer (provided) was added and the plate was incubated for 60 minutes at room temperature. The plate was washed five times with 200 µl per well of 1X PBS-0.005% ($^{v}/_{v}$) Tween-20. Leaving the substrate blank wells empty, 100 µl of IL-8 standards, in the concentration range of: 1, 2.5, 6.1, 15.4, 38.4, 96 and 240 pg/ml and 100 µl of cell culture supernatants diluted 1 in 10 with MEM-ITS were added. The plate was covered and incubated for 60 minutes at room temperature. The plate was washed five times with 200 µl per well of 1X PBS-0.005% ($^{v}/_{v}$) Tween-20. Leaving the substrate blank wells empty, 100 µl of biotinylated sheep antibody to human IL-8, diluted 1 in 100 with dilution buffer (provided) was added to each well and the plate was covered and incubated for 60 minutes at room temperature. The plate was washed five times with 200 µl per well of 1X PBS-0.005% ($^{v}/_{v}$) Tween-20. Leaving the substrate blank wells empty. Streptavidin-HRP conjugate was diluted 1 in 10,000 in dilution buffer (provided) and 100 µl was added to each well. The plate was covered and incubated at room temperature for 30 minutes. Immediately before use, substrate solution was prepared: 12 ml substrate buffer (0.11 M acetate buffer pH 5.5), 200 µl of tetramethylbenzidine (TMB, 6 mg/ml in DMSO: Sigma, UK) and 12 µl H_2O_2 (3% H_2O_2 in deionised water: Sigma, UK). The plate was washed five times by adding 200 µl per well of 1X PBS-0.005% ($^{v}/_{v}$) Tween-20. To all wells including substrate blank wells, 100 µl of the resultant substrate solution was added and the plate was covered and incubated for 30 minutes at room temperature, protected from light. In order to stop the reaction, 100 µl of stop solution (1.8 M H_2SO_4) was added to each well and the optical density at 450 nm was determined for each well using a microplate reader (Dynex MRX: West Sussex, UK). IL-8 concentration in the samples was determined from the standard curve, taking into account the 1 in 10 dilution.

3.3.9. Stimulation of 16HBE 14o^{-} cells with neutrophil elastase

A concentration range of: 0, 0.1, 0.03, 0.1, 0.3 and 0.5 U/ml neutrophil elastase (3 U/ml stock dissolved in 1X PBS: Sigma, Dorset, UK) was added to cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. Cells were then

mechanically wounded (W8) as described in *section 2.2.5* and incubated for 20 minutes and 2 hours at 37°C. Cell culture supernatants were then harvested as described in *section 2.2.4* and the concentration of fibrinogen and FXIII was determined by immunoblot as described in *section 3.3.3.*

3.4. Results

3.4.1. TF

3.4.1.1. Immunohistochemistry for TF

TF is an integral membrane glycoprotein and is important in the initiation of the coagulation cascade. It is believed that TF is constitutively expressed by cells that play a vital role in repair following injury (Osterud, 1997), therefore, its presence was investigated in the intact and wounded monolayers of 16HBE 14o^{-} cells.

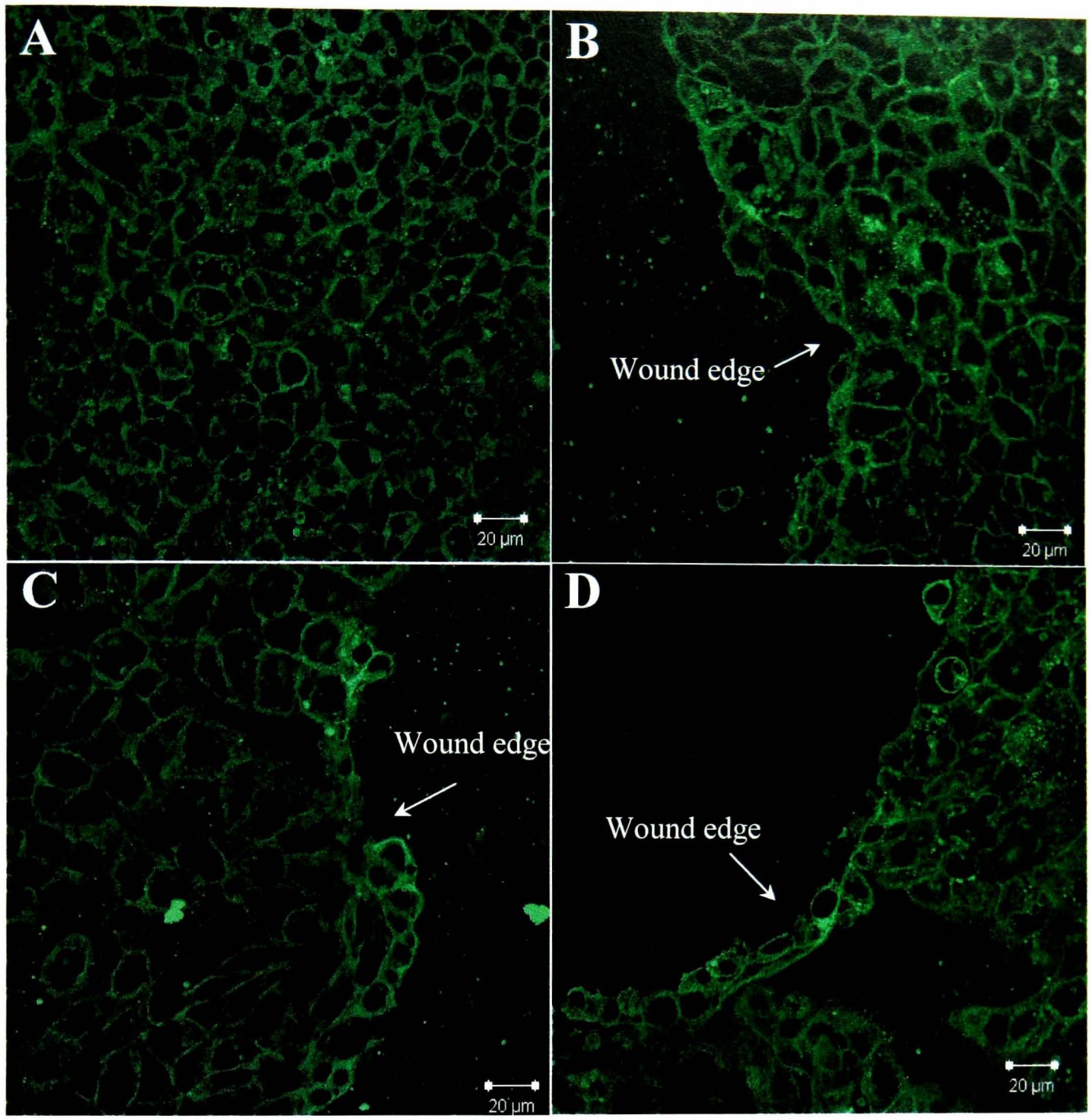

Figure 3.8. Fluorescent immunolocalisation of TF on 16HBE 14o$^-$ cells visualised by confocal laser scanning microscopy (CLSM) at x400 magnification. Scale bar represents 20 µm. (A) represents an unwounded monolayer of 16HBE 14o$^-$ cells. (B) 30 minutes *post*-wound. (C) 1 hour *post*-wound. (D) 2 hour *post*-wound.

The image represented by *figure 3.8 A* shows positive immunostaining for TF in the unwounded cell monolayer. Consistent with the literature, the strong staining at the periphery of the cell indicates that TF is a trans-membranous protein but more importantly, it shows that TF is constitutively expressed by 16HBE 14o$^-$ cells. At 1 and 2 hours *post*-wound (*figures 3.8 C and 3.8 D* respectively), there is evidence of TF upregulation at the wound edge suggesting involvement of the protein in wound repair. Moreover, *figure 3.9 B* demonstrates polarised cells with pseudopodia projecting in the direction of the wound. Immunostaining for TF is also evident in vesicles within the

cells at the edge of the wound, implying that TF may be pre-formed and subsequently inserted into the membrane following wounding.

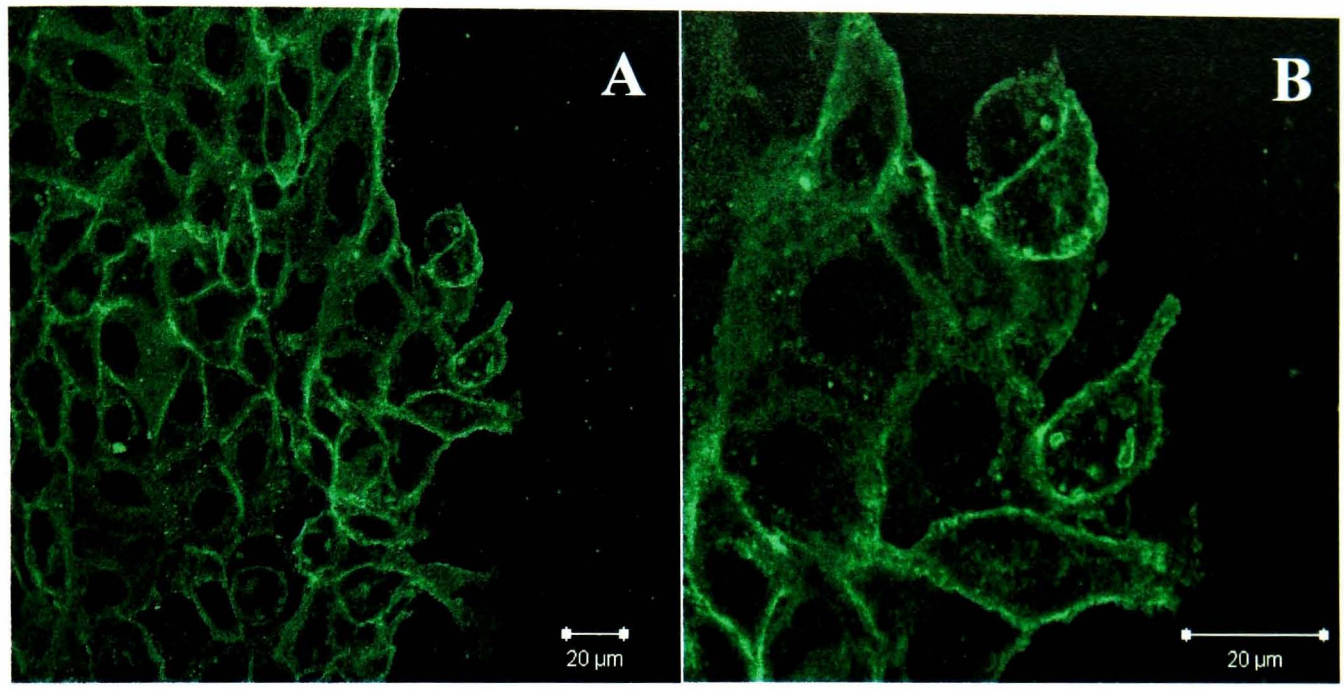

Figure 3.9. Fluorescent immunolocalisation of TF on 16HBE 14o$^-$ cells visualised by CLSM at x400 magnification. Scale bar represents 20 µm. (A) 2 hours *post*-wound. (B) 16HBE 14o$^-$ cells at the wound edge with pseudopodia projecting towards the centre of the wound, indicative of directed migration.

In order to verify that the immunostaining for TF was specific, negative controls were included, whereby staining was investigated in the absence of primary antibody and in the absence of secondary antibody (*figure 3.10*).

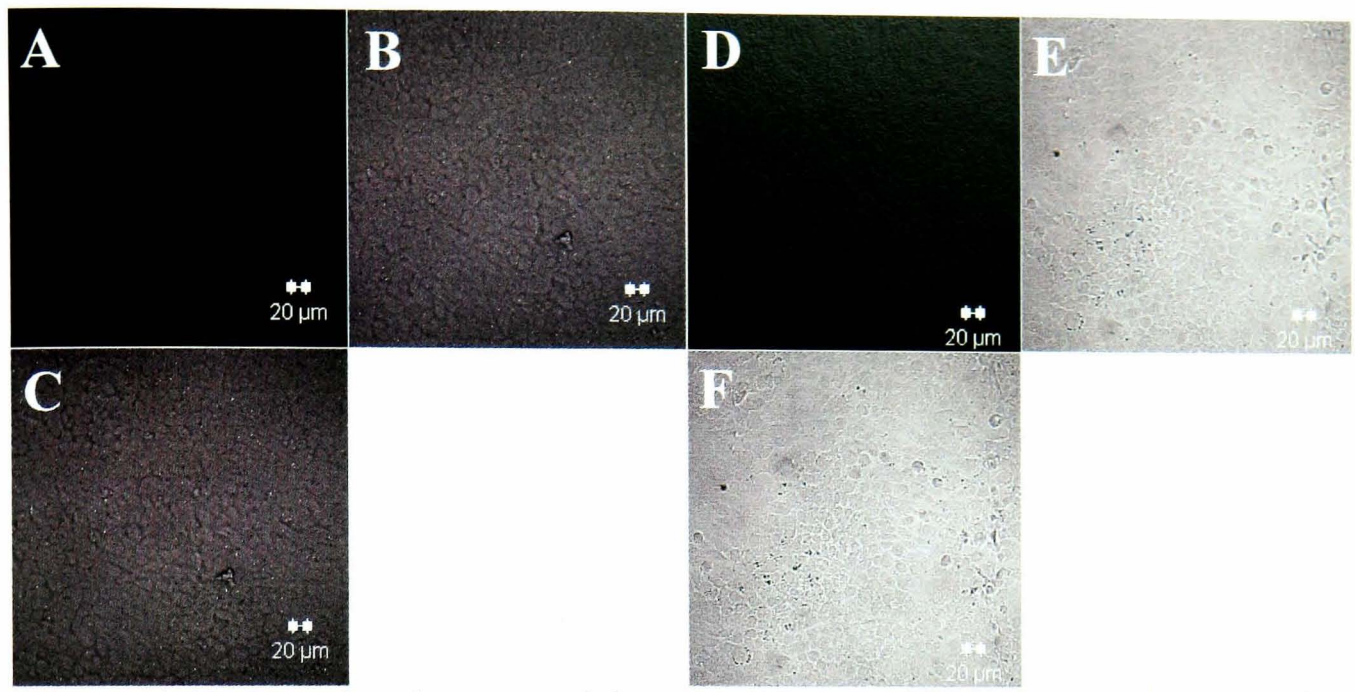

Figure 3.10. Negative immunostaining for TF on 16HBE 14o$^-$ cells. Scale bar represents 20 µm. (A) No fluorescence for TF on 16HBE 14o$^-$ cells in the absence of primary antibody. (B) Differential interference contrast (DIC) image of the unwounded monolayer of 16HBE 14o$^-$ cells. (C) Combination of images A and B. (D) No fluorescence for TF on 16HBE 14o$^-$ cells in the absence of secondary antibody. (E) DIC image of the unwounded monolayer of 16HBE 14o$^-$ cells. (F) Combination of images D and E.

Negative controls displayed by DIC images of the unwounded cell monolayer indicate that in the absence of primary antibody (i.e. secondary antibody alone, *figures 3.10 A, B and C*) and in the absence of secondary antibody (i.e. primary antibody alone, *figures 3.10. D, E and F*), there is no immunostaining for TF.

3.4.1.2. TF activity

Since immunohistochemistry demonstrated constitutive expression of TF by 16HBE 14o$^-$ cells, it was of interest to measure the activity of TF, at baseline and in response to wounding.

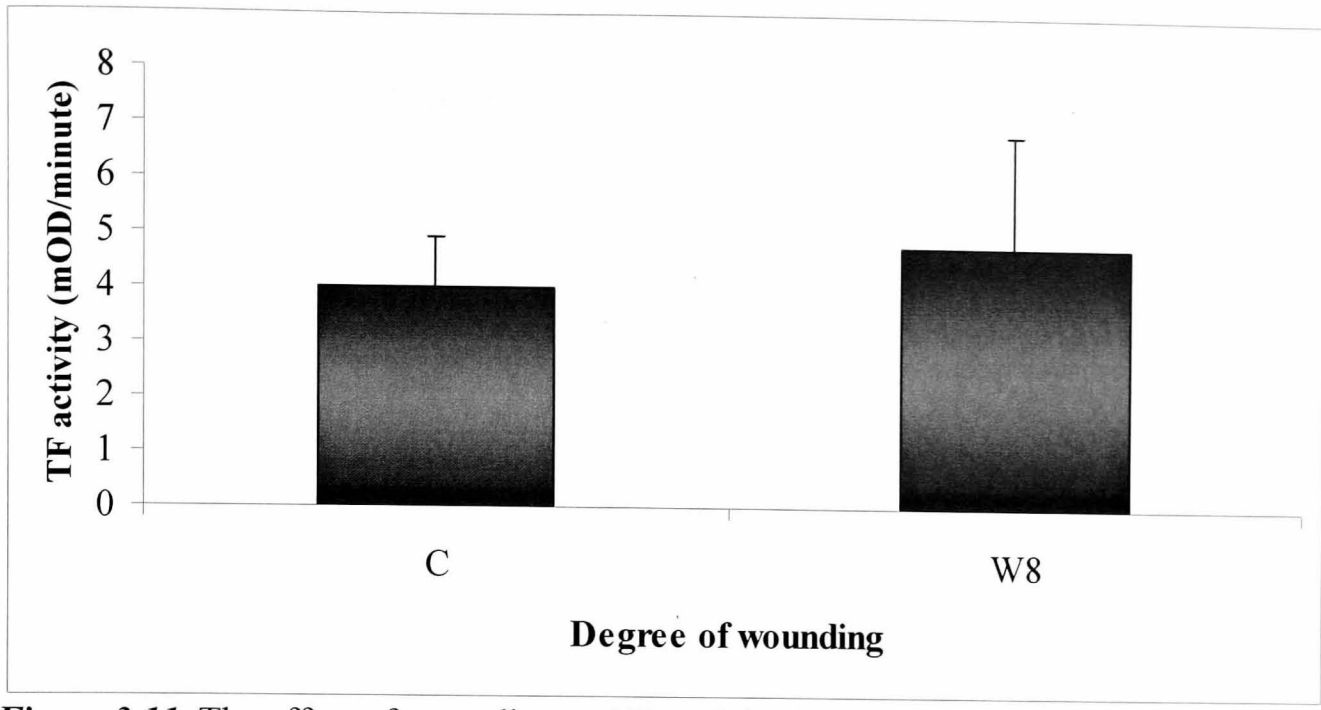

Figure 3.11. The effect of wounding on TF activity in 16HBE 14o$^-$ cells. Data represent mean ± SEM of replicates of 5 and representative of two experiments.

TF activity at baseline was 4.0 ± 0.89 mOD/minute compared to 4.8 ± 2.0 mOD/minute in the wounded cell cultures of 16HBE 14o$^-$ cells. The effect of wounding on TF activity was not significant.

3.4.2. Effect of wounding on the release of coagulation factors

3.4.2.1. Fibrinogen

Figure 3.12 represents a typical standard curve for the detection of fibrinogen in cell culture supernatants. A linear relationship between immunoblot density and fibrinogen concentration measured in arbitrary units (AU) was demonstrated up to 20 μg/ml. At higher concentrations, the density values tended to plateau, therefore samples were analysed in the linear range of 0-20 μg/ml.

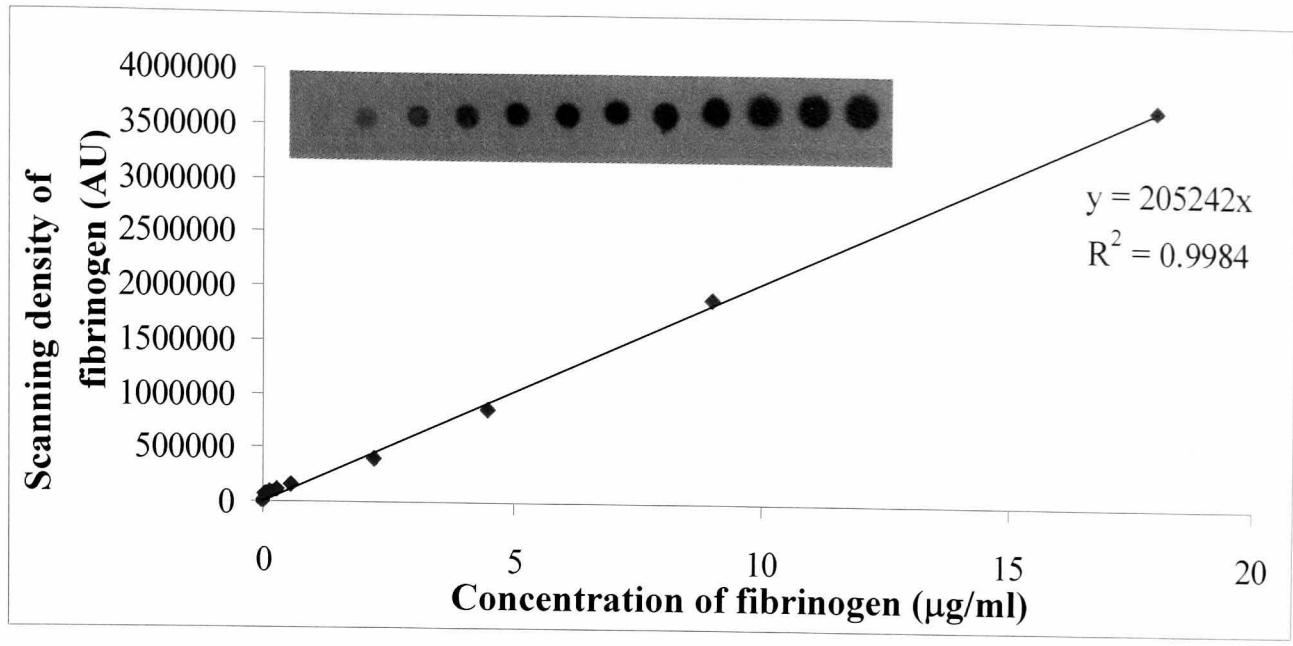

Figure 3.12. Representative graph illustrating the standard curve of fibrinogen. A serial dilution of normal plasma was used as a reference for fibrinogen concentration.

Figure 3.13. Illustrates the release of fibrinogen into cell culture supernatants at 2 hours *post*-wounding determined by immunoblot analysis.

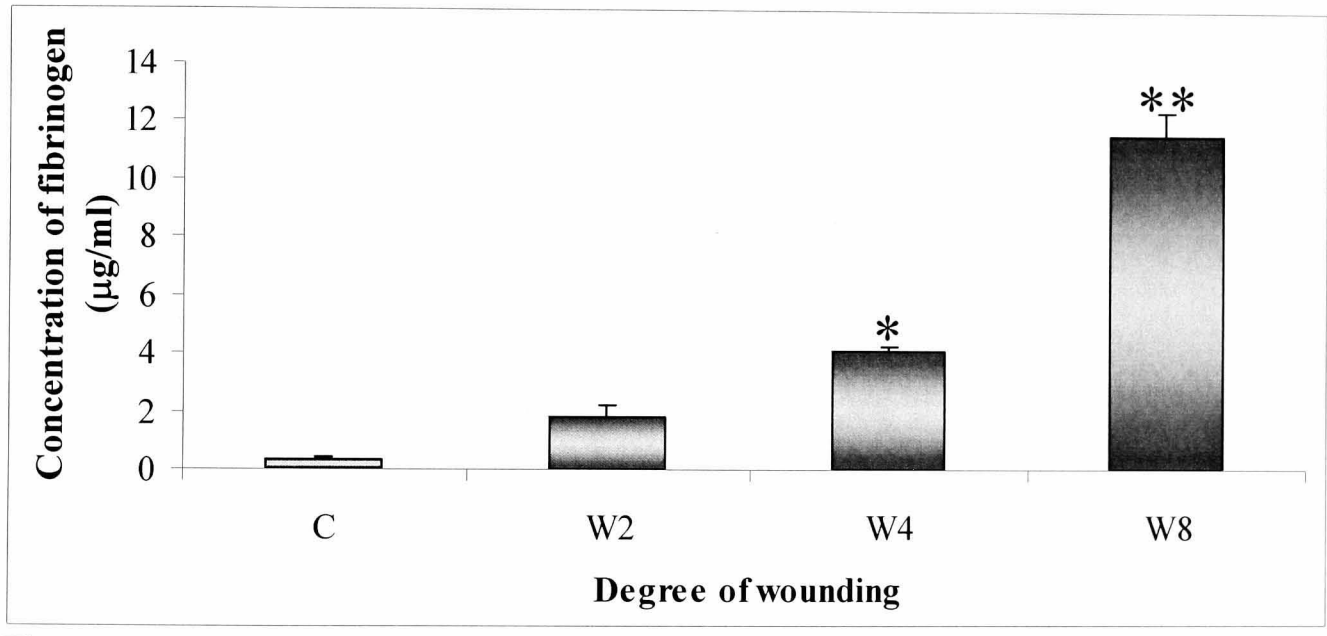

Figure 3.13. Effect of wounding on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. C=unwounded cells, W2=2 wounds, W4=4 wounds and W8=8 wounds. Data represent mean ± SEM (*n=3*). * Indicates $P<0.05$ and ** indicates $P<0.001$ compared to the unwounded control.

Cell culture supernatants were removed from 16HBE 14o$^-$ cells 2 hours *post*-wounding and analysed for fibrinogen. Supernatants displayed a significant increase in the concentration of soluble fibrinogen with the degree of wounding (W4=4.08 ± 0.20 µg/ml and W8=11.58 ± 0.78 µg/ml) compared to the unwounded cell control (C=0.35 ± 0.08 µg/ml), indicating that there is release of fibrinogen that is dependent on the extent of wounding.

3.4.2.2. FXIIIA

The cellular form of coagulation FXIII represents a dimer of two potentially active A subunits (FXIIIA) (Katona *et al.*, 2005). Therefore, an antibody that recognises the FXIIIA subunit was used, since this subunit represents the cell associated form.

Figure 3.14 represents a typical standard curve for the detection of FXIIIA in cell culture supernatants. A linear relationship between immunoblot density and FXIIIA concentration measured in AU was demonstrated up to 4 ng/ml. At higher concentrations, the density values tended to plateau, therefore samples were analysed in the linear range of 0-4 ng/ml.

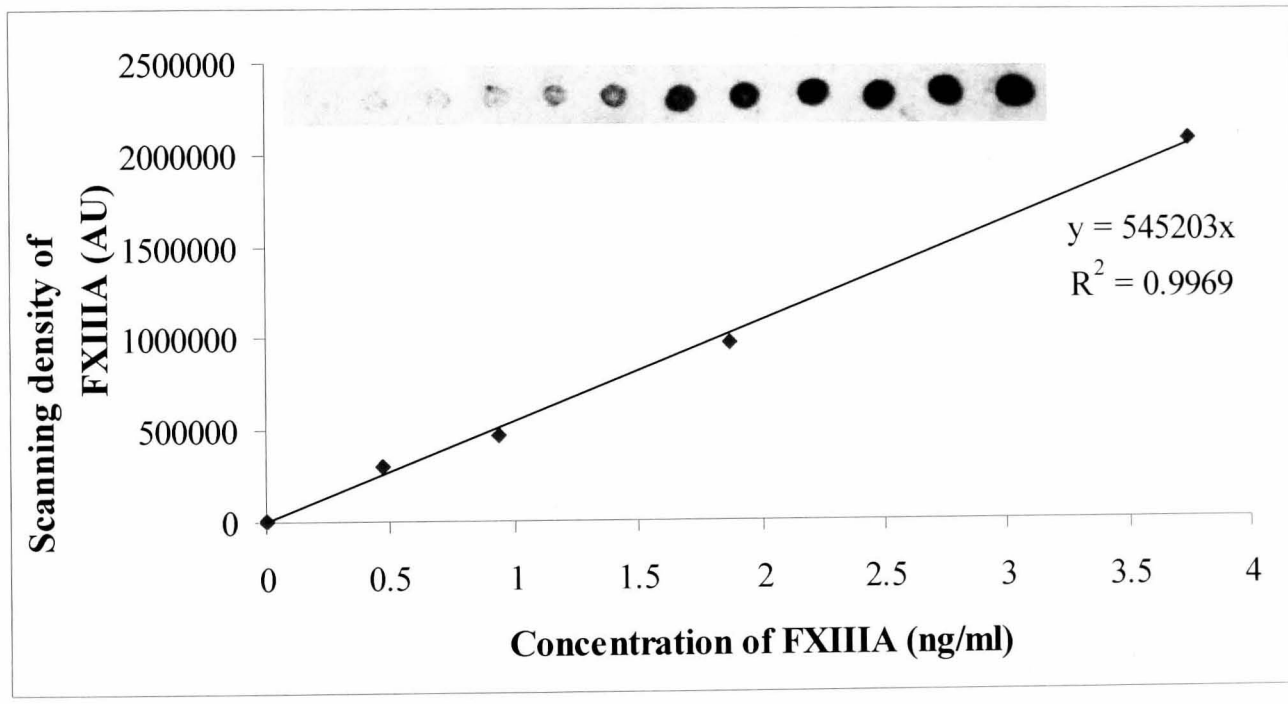

Figure 3.14. Representative graph illustrating the standard curve for FXIIIA. A serial dilution of normal plasma was used as a reference of FXIIIA concentration.

Figure 3.15 Illustrates the release of FXIIIA into cell culture supernatants at 2 hours *post*-wounding determined by immunoblot analysis.

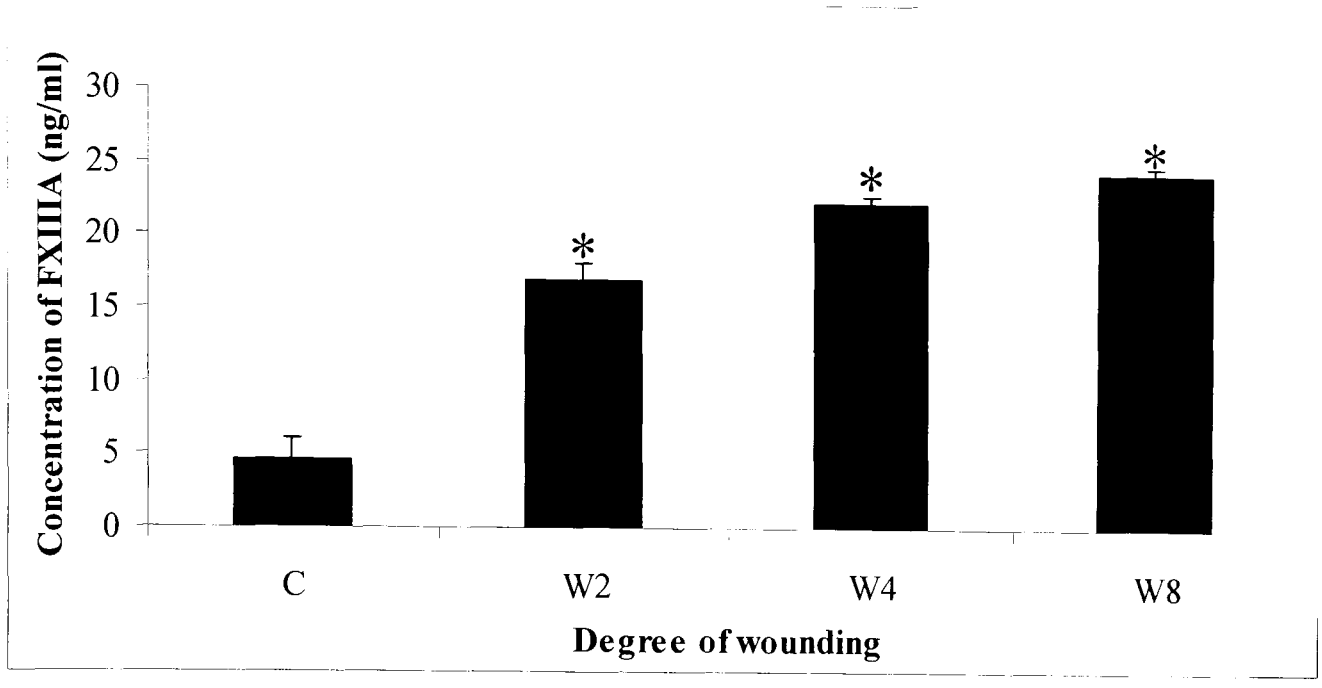

Figure 3.15. Effect of wounding on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. C=unwounded cells, W2=2 wounds, W4=4 wounds and W8=8 wounds. Data represent mean ± SEM (*n=3*). * Indicates $P<0.05$ compared to the unwounded control.

Cell culture supernatants were removed from 16HBE 14o$^-$ cells 2 hours *post*-wounding and analysed for FXIIIA. Supernatants displayed a significant increase in FXIII concentration with the degree of wounding (W2=17.00 ± 1.06 ng/ml, W4=22.30 ± 0.51 ng/ml and W8=24.43 ± 0.59 ng/ml) compared to the unwounded cell control (C=4.49 ± 1.50 ng/ml) at 2 hours, indicating that FXIII was released from cells with the extent of cell damage.

3.4.2.3. D-dimers

D-dimer concentration, a measure of both fibrin formation and breakdown was measured in cell culture supernatants from intact and wounded cell layers. *Figure 3.16* represents a typical standard curve for the detection of D-dimers in cell culture supernatants. A linear relationship between immunoblot density and D-dimer concentration measured in AU was demonstrated up to 70 ng/ml. At higher concentrations, the density values tended to plateau, therefore samples were analysed in the linear range of 0-70 ng/ml.

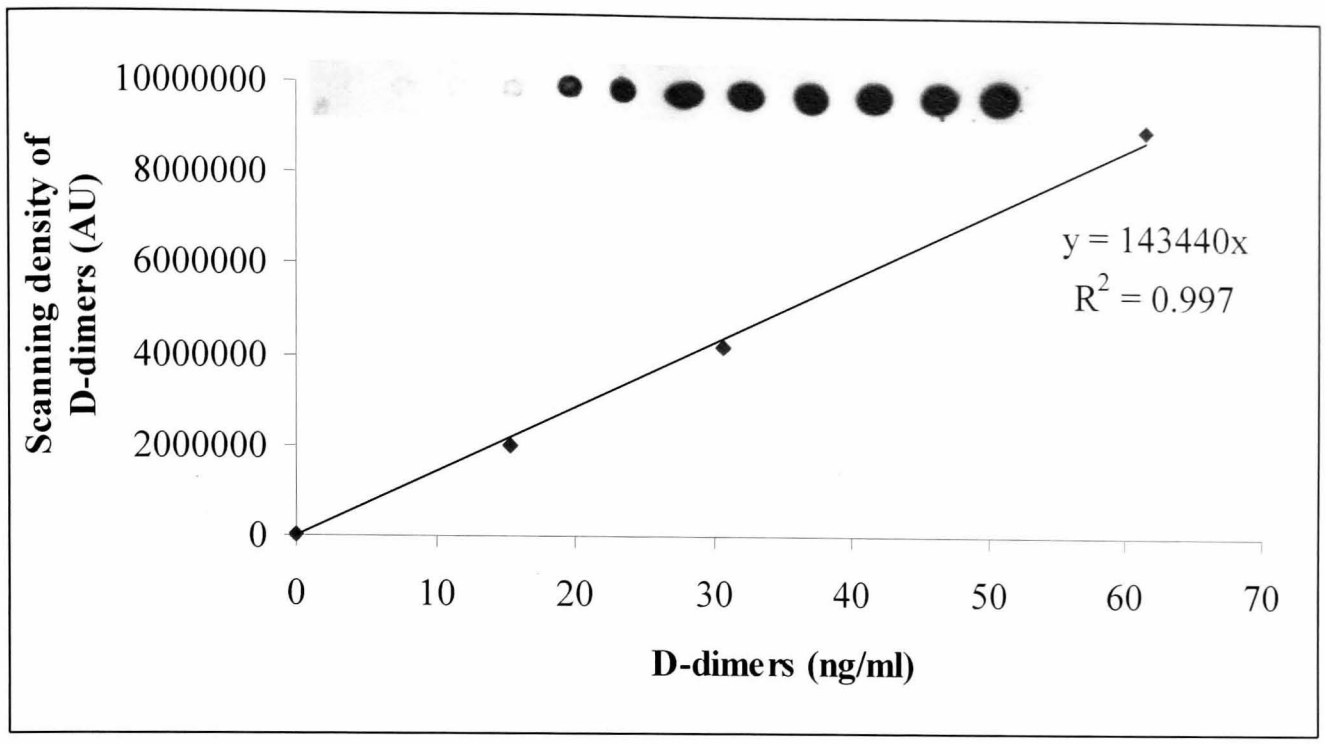

Figure 3.16. Representative graph illustrating the standard curve for D-dimers. A high molecular weight D-dimer standard was used as a reference of D-dimer concentration.

Figure 3.17 illustrates the release of D-dimers into cell culture supernatants at 2 hours *post*-wounding determined by immunoblot analysis.

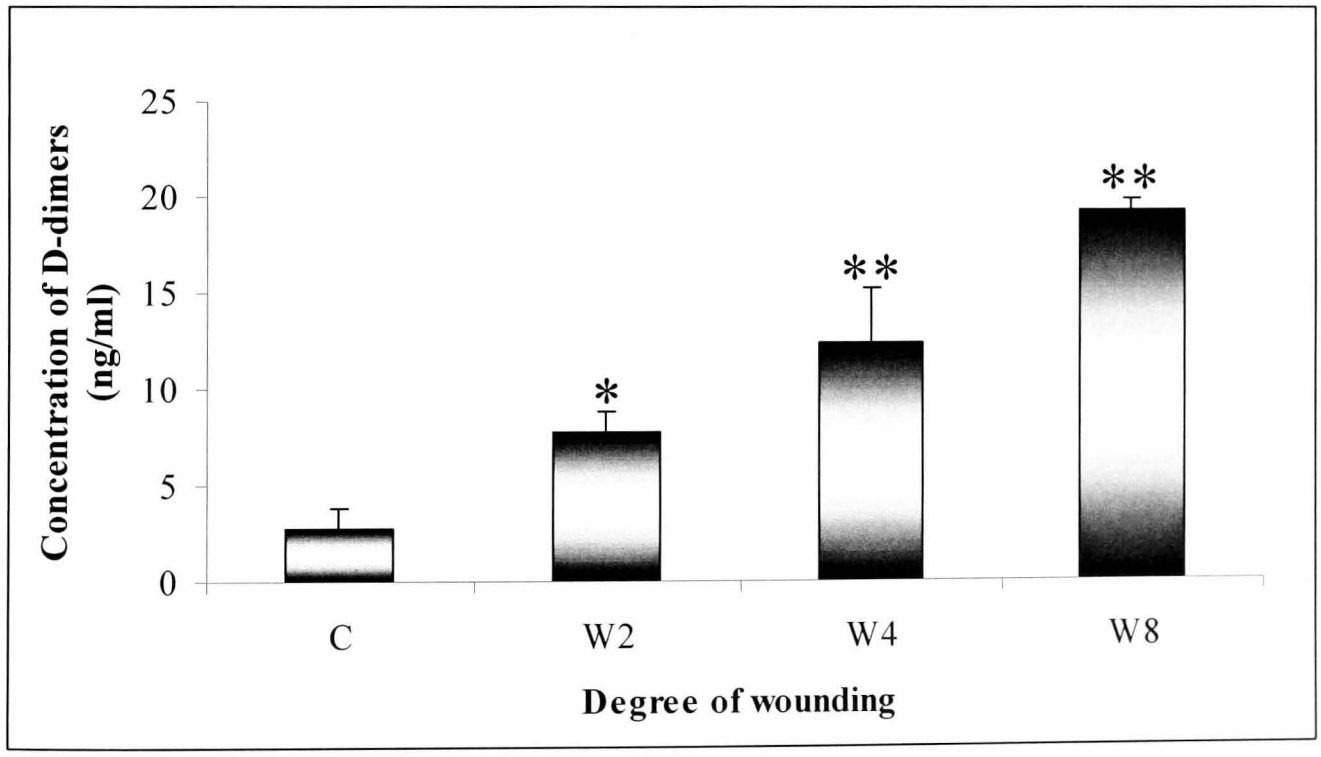

Figure 3.17. Effect of wounding on the concentration of D-dimers in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. C=unwounded cells, W2=2 wounds, W4=4 wounds and W8=8 wounds. Data represent mean ± SEM (*n=3*). * Indicates $P<0.05$ and ** indicates $P<0.01$ compared to C.

Cell culture supernatants were removed from 16HBE 14o$^-$ cells 2 hours post-wounding and analysed for D-dimers. Supernatants displayed a significant increase in the concentration of D-dimers with increasing cell damage (W2=7.79 ± 1.01 ng/ml, W4=12.40 ± 0.58 ng/ml and W8=19.28 ± 0.58 ng/ml) compared to the unwounded cell control (C=2.77 ± 1.04 ng/ml) indicating that wounding stimulated an increase in the release of D-dimers from the cells into cell culture supernatants.

3.4.3. Effect of wounding on LDH levels

Supernatants and cell lysates were assayed for LDH to determine whether the release of coagulation factors in response to wounding was due to cytotoxicity as a consequence of the rupturing of the dislodged cells by mechanical wounding.

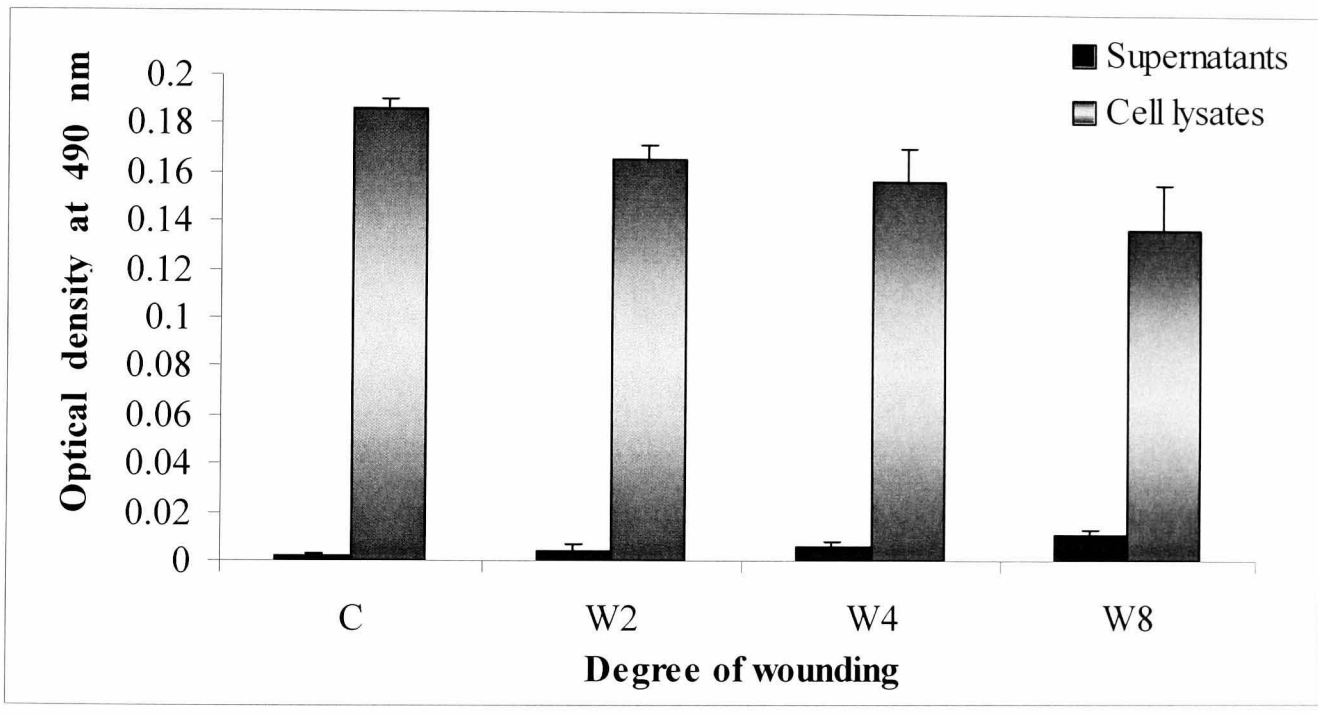

Figure 3.18. LDH levels in cell culture supernatants and cell lysates of 16HBE 14o$^-$ cells at 2 hours post-wounding. C=unwounded cells, W2=2 wounds, W4=4 wounds and W8=8 wounds. Data represent mean ± SEM (*n=3*).

Analysis of LDH levels in cell culture supernatants revealed a small but non-significant increase, dependent on the extent of wounding (C=0.002 ± 0.001, W2=0.004 ± 0.002, W4=0.006 ± 0.002 and W8=0.01 ± 0.002). This correlated to a small, non-significant decrease of LDH in the cell lysates dependent on the extent of wounding (C=0.19 ± 0.004, W2=0.17 ± 0.006, W4=0.16 ± 0.01 and W8=0.14 ± 0.02). In the unwounded

cells, supernatants displayed 1% LDH relative to the total cell number and this proportion was unaltered with the extent of wounding.

3.4.4. Growth Factors

Since growth factors have been implicated in wound repair, their expression was investigated in this model of 16HBE $14o^-$ wound repair.

3.4.4.1. EGF

It is well established that EGF is an important regulator of bronchial epithelial restitution *in vivo.* Weak expression of EGF has been demonstrated in the bronchial epithelium and the epithelium has also been shown to express the EGFR. Therefore, the involvement of EGF was investigated in wound repair by analysis of EGF concentration in cell culture supernatants up to 12 hours *post*-wounding. The assay was highly sensitive, with a detection limit of 0.7 pg/ml. *Figure 3.19* illustrates the standard curve for EGF. A linear relationship between the optical density and the concentration of EGF was seen up to 250 pg/ml; therefore the concentration of EGF in the cell culture supernatants was determined within the range of 0-250 pg/ml.

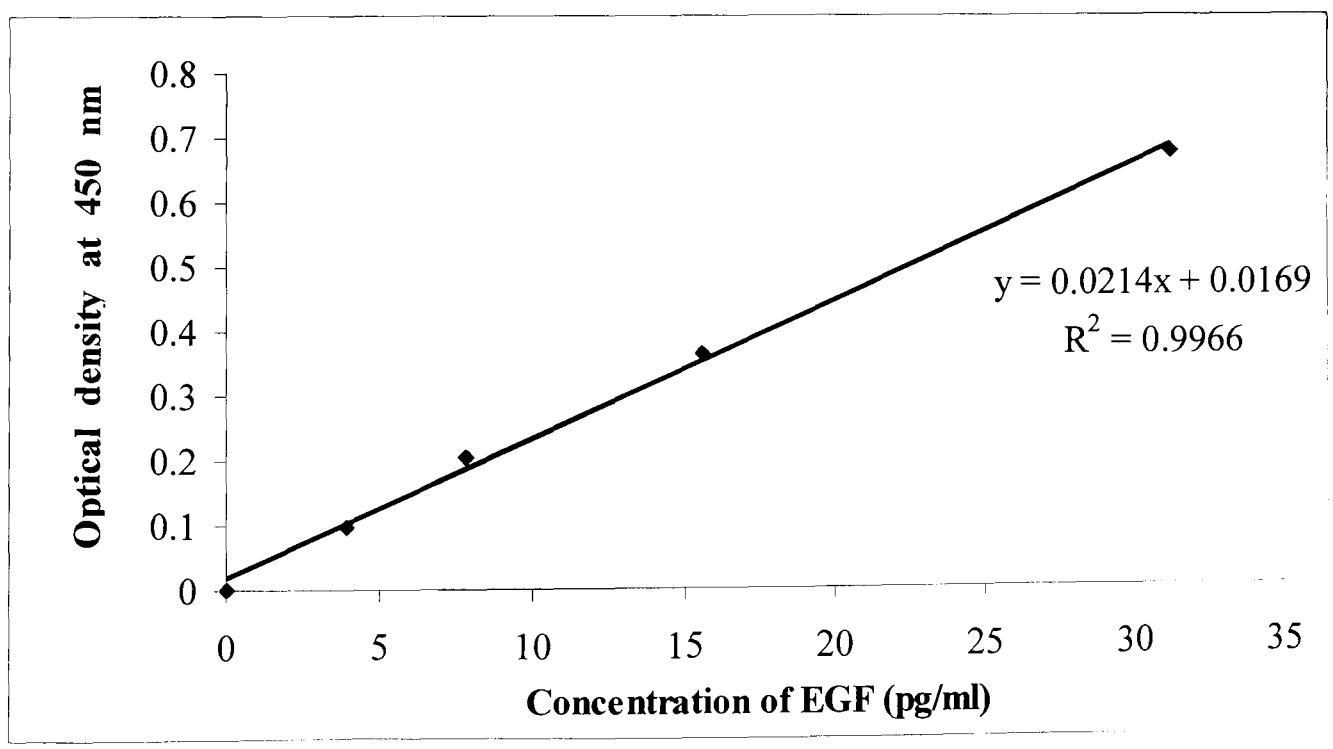

Figure 3.19. Representative graph illustrating the standard curve for EGF.

Time (h) *post*-wound	Mean optical density (OD) at 450 nm	Mean concentration of EGF (pg/ml)	SEM
UW	0.004	0.123	0.007
2	0.005	0.256	0.006
4	0.003	0.025	0.005
6	0.003	0.059	0.020
8	0.003	0.085	0.009
10	0.004	0.016	0.011
12	0.003	0.063	0.003

Table 3.2. Optical density values indicating the effect of time *post*-wounding on the concentration of EGF in cell culture supernatants from 16HBE 14o$^-$ cells. UW=unwounded cells. Data represent mean ± SEM (n=5).

The values illustrated in *table 3.2* indicate that the concentration of EGF released into cell culture supernatants at baseline and in response to wounding was extremely low. Furthermore, there was no statistically significant difference between the wounded cell cultures at any of the time-points investigated compared to the unwounded cell control.

3.4.4.2. KGF

KGF is a member of the fibroblast growth factor family and has been shown to target the bronchial epithelium and promote repair. Thus, the involvement of KGF was investigated in wound repair by analysis of KGF concentration in cell culture supernatants up to 12 hours *post*-wounding. The assay was highly sensitive, with a minimal detectable concentration of 15 pg/ml KGF. *Figure 3.20* illustrates the standard curve for KGF. A linear relationship between the optical density and the concentration of KGF was seen up to 2000 pg/ml; therefore the concentration of KGF in the cell culture supernatants was determined within the range of 0-2000 pg/ml.

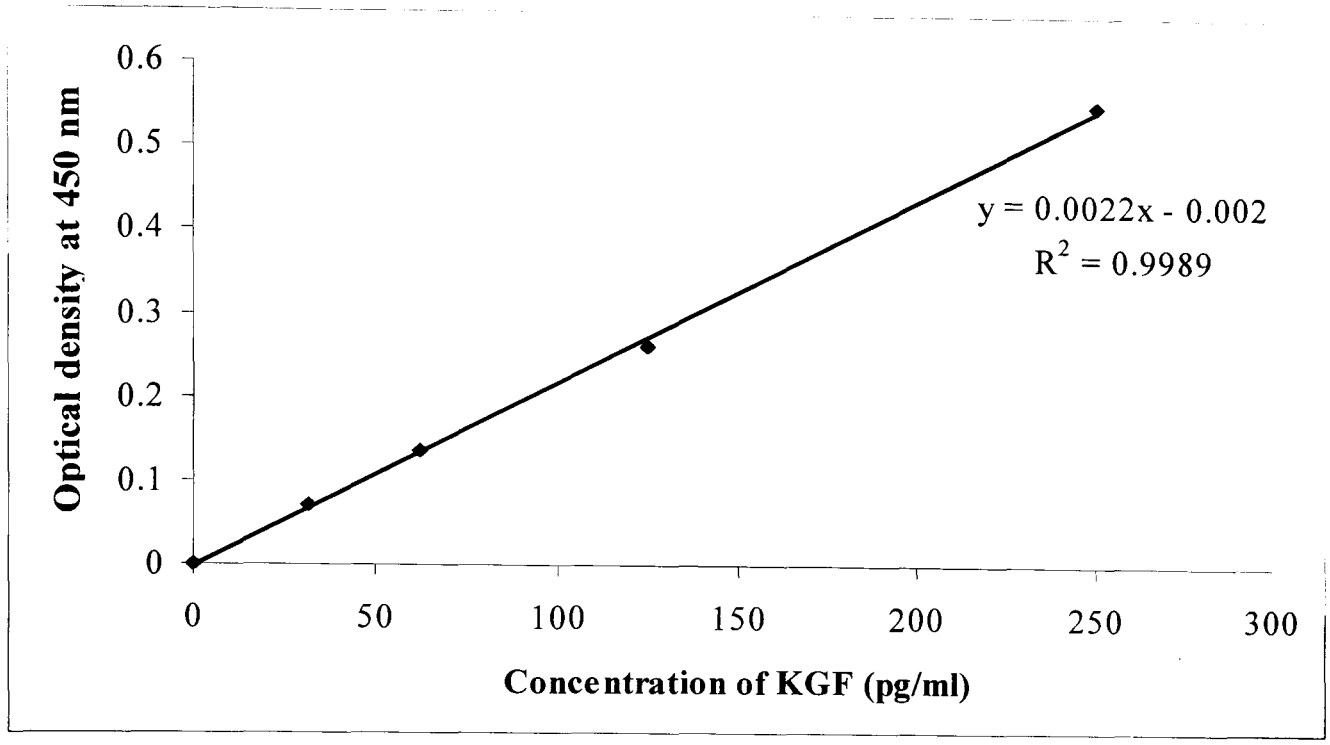

Figure 3.20. Representative graph illustrating the standard curve for KGF.

Time (h) *post*-wound	Mean optical density (OD) at 450 nm	Mean concentration of KGF (pg/ml)	SEM
UW	0.017	13.862	2.206
2	0.012	10.111	4.661
4	0.012	10.111	3.442
6	0.013	10.945	4.955
8	0.014	11.445	4.836
10	0.015	12.611	2.971
12	0.016	12.861	2.974

Table 3.3. Optical density values indicating the effect of time *post*-wounding on the concentration of KGF in cell culture supernatants from 16HBE $14o^-$ cells. UW=unwounded cells. Data represent mean ± SEM ($n=5$).

Similarly, the analysis of cell culture supernatants for KGF revealed that the concentration of this growth factor in supernatants of unwounded cells and those investigated at time intervals *post*-wounding was also very low with reference to the standard curve for KGF and there was no significant difference between any of the conditions measured.

3.4.4.3. TGF-β1

Since TGF-β1 has been shown to be synthesised by epithelial cells and has been implicated in repair of 16HBE 14o$^-$ cell monolayers in culture (Howat *et al.*, 2002), its role was investigated in this model of 16HBE 14o$^-$ wound repair by analysis of total and active concentration of TGF-β1 in cell culture supernatants up to 12 hours *post*-wounding. The assay was highly sensitive, with a mean minimal detectable concentration of 4.61 pg/ml total TGF-β1. *Figure 3.21* illustrates the standard curve for TGF-β1. A linear relationship between the optical density and the concentration of TGF-β1 was seen up to 2000 pg/ml; therefore the concentration of TGF-β1 in the cell culture supernatants was determined within the range of 0-2000 pg/ml. Total TGF-β1 refers to the concentration of TGF-β1 as a consequence of acid-activation and includes both latent and active TGF-β1; and active TGF-β1 refers to the concentration of TGF-β1 in samples before acid-activation. The concentration of latent TGF-β1 may be calculated from the active and total concentrations.

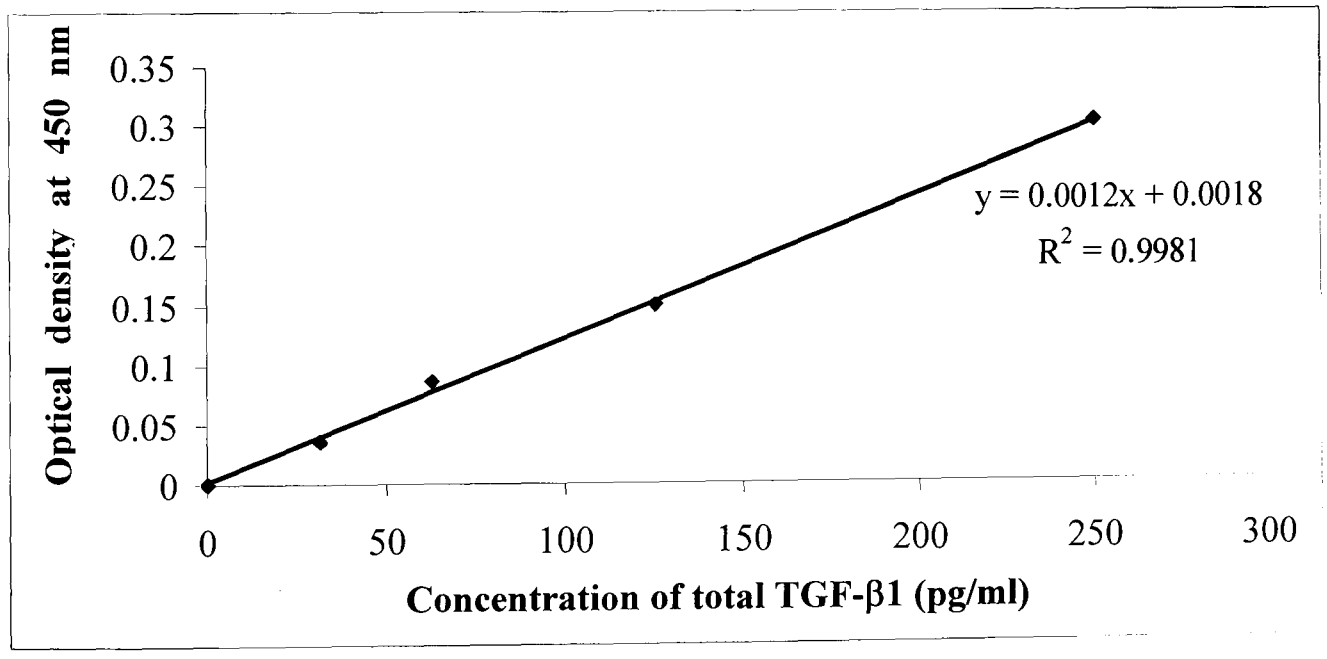

Figure 3.21. Representative graph illustrating the standard curve for TGF-β1.

Time (h) *post*-wound	Mean optical density (OD) at 450 nm	Mean concentration of total TGF-β1 (pg/ml)	SEM
UW	0.015	16.753	1.680
2	0.018	19.698	1.780
4	0.027	30.309 *	5.389
6	0.027	30.309 *	4.595
8	0.029	31.809 *	6.356
10	0.029	32.531 *	3.285
12	0.027	29.975 *	2.773

Table 3.4. Optical density values indicating the effect of time *post*-wounding on the concentration of latent TGF-β1 in cell culture supernatants from 16HBE 14o$^-$ cells. UW=unwounded cells. Data represent mean ± SEM (*n=5*). * Indicates P<0.005 compared to UW.

Time (h) *post*-wound	Mean optical density (OD) at 450 nm	Mean concentration of active TGF-β1 (pg/ml)	SEM
UW	0.011	13.650	3.840
2	0.011	13.300	3.302
4	0.007	8.80	1.880
6	0.009	11.200	1.823
8	0.011	14.025	4.474
10	0.015	16.600	3.292
12	0.014	16.975	2.268

Table 3.5. Optical density values indicating the effect of time *post*-wounding on the concentation of active TGF-β1 in cell culture supernatants from 16HBE 14o$^-$ cells. UW=unwounded cells. Data represent mean ± SEM (*n=5*).

Wounded cell cultures displayed higher concentrations of TGF-β1 in its total form compared to active TGF-β1, although concentrations were found to be very low. However, there was a significant increase in the total concentration of TGF-β1 in cell culture supernatants 4 hours *post*-wound (30.31 ± 5.39 pg/ml) compared to 16.75 ± 1.68 pg/ml in the unwounded cell cultures. This effect was maintained up to 12h *post*-wounding (6 hours = 30.31 ± 4.60 pg/ml; 8 hours = 31.81 ± 3.29 pg/ml; 10 hours = 32.53 ± 3.29 pg/ml; 12 hours = 29.98 ± 2.77 pg/ml). This increase was associated with

an increase in latent TGF-β1 since there was no effect of wounding on the concentration of active TGF-β1 in cell culture supernatants.

Time (h) *post-* wound	Mean concentration of total TGF-β1 (pg/ml)	Mean concentration of active TGF-β1 (pg/ml)	Mean concentration of latent TGF-β1 (pg/ml)
UW	16.753	13.650	**3.10**
2	19.698	13.300	**6.40**
4	30.309 *	8.80	**21.51**
6	30.309 *	11.200	**19.12**
8	31.809 *	14.025	**17.78**
10	32.531 *	16.600	**15.93**
12	29.975 *	16.975	**13.00**

Table 3.6. Mean concentration of latent TGF-β1 calculated from the active and total concentrations.

3.4.4.4. HGF and *c-Met* receptor

HGF is synthesised by mesenchymal cells and has been implicated in bronchial epithelial repair by enhancing cell migration *in vitro*. HGF and corresponding HGF (*c-Met*) receptor expression was therefore investigated in this model of 16HBE 14o$^-$ cells.

HGF was not detected by immunohistochemistry of 16HBE 14o$^-$ cells or by ELISA (data not shown). However, the *c-Met* receptor was expressed by these cells (*figure 3.22 A*), and its expression was upregulated in response to 100 ng/ml HGF (*figure 3.22.B*) and in response to wounding (*figure 3.22 C*).

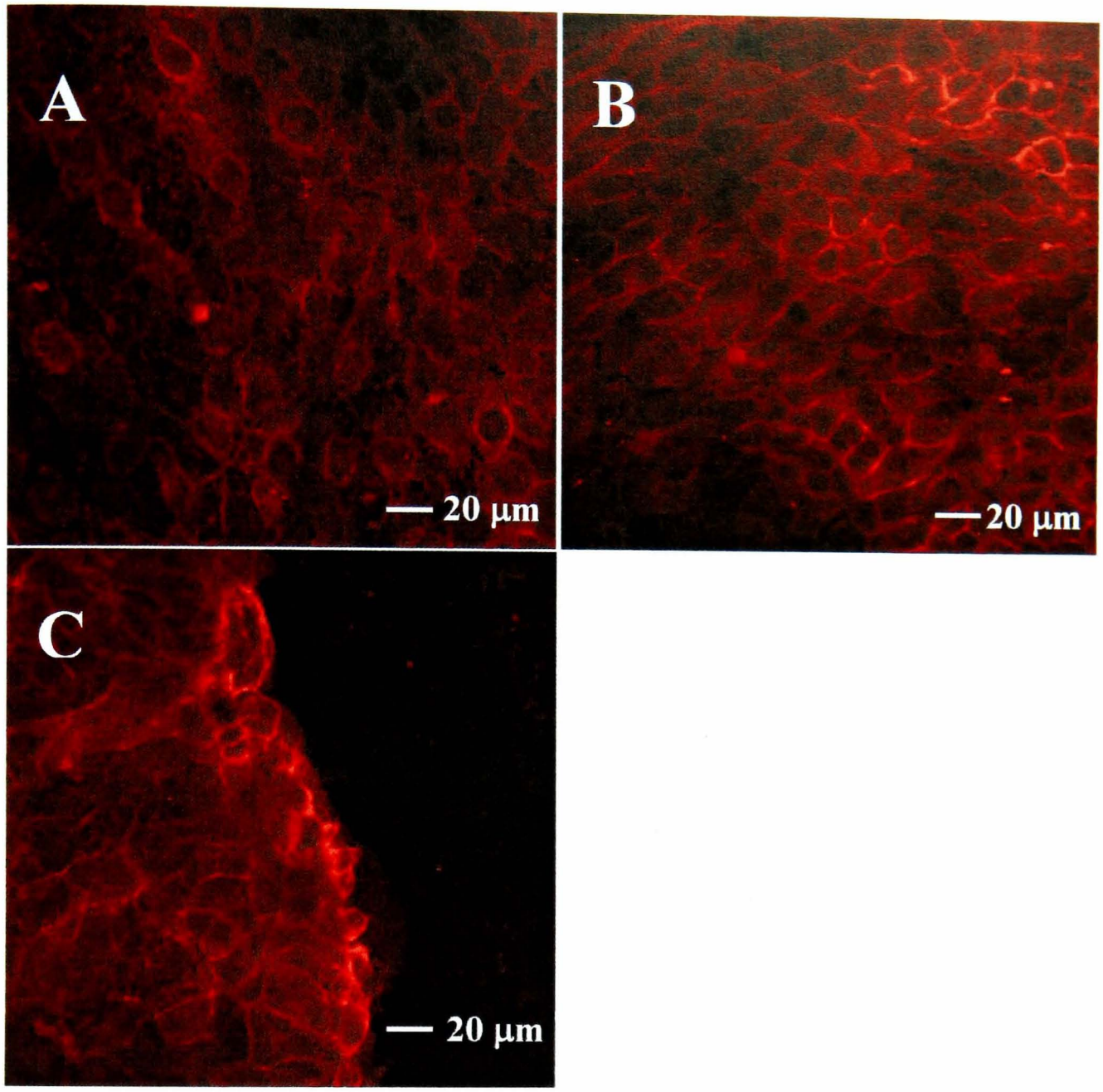

Figure 3.22. Fluorescent immunolocalisation of c-Met receptor on 16HBE 14o$^-$ cells visualised by CLSM at x400 magnification. Scale bar represents 20 μm. (A) represents an unstimulated monolayer of 16HBE 14o$^-$ cells. (B) An intact monolayer of 16HBE 14o$^-$ cells stimulated with 100 ng/ml HGF (C) A wounded monolayer of unstimulated 16HBE 14o$^-$ cells. Images are representative of two separate experiments.

3.4.4.5 Effect of HGF on FXIIIA expression

Since HGF has been implicated in wound repair, it was of interest to investigate its effects on expression of FXIIIA, the transglutaminase cross-linking enzyme that is important in fibrinogenesis.

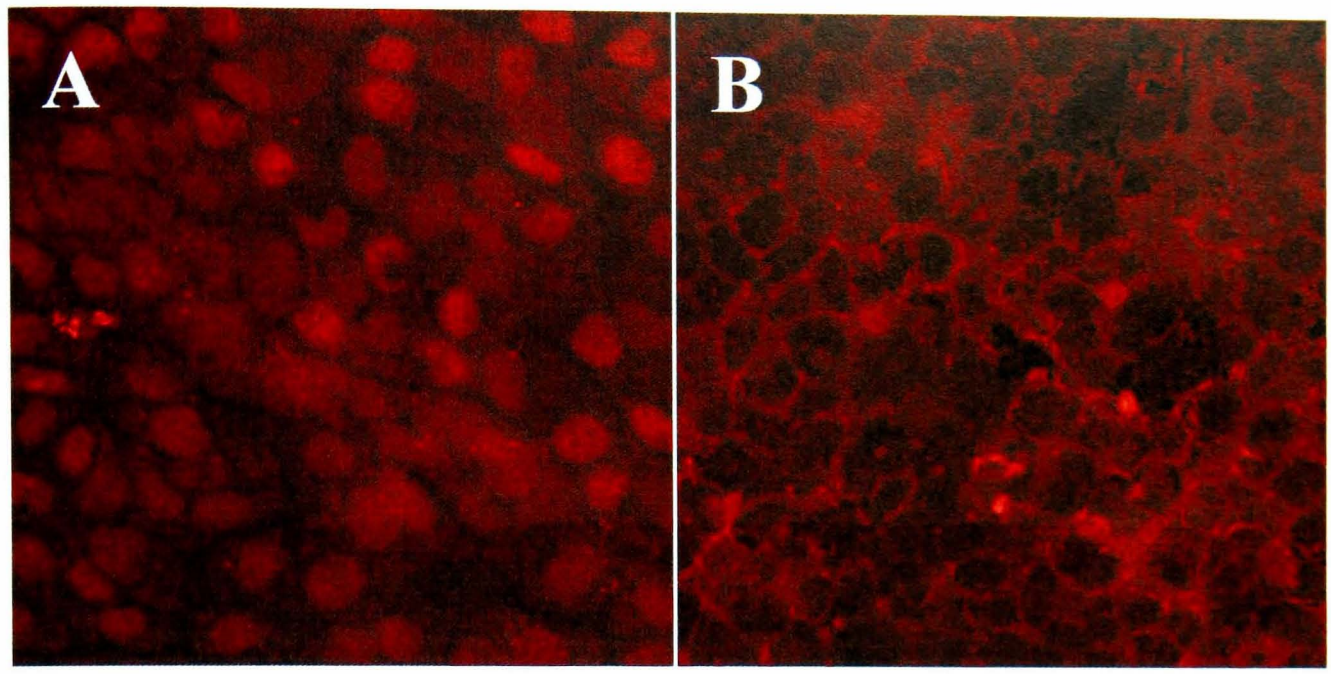

Figure 3.23. Fluorescent immunolocalisation of FXIIIA on 16HBE 14o$^-$ cells visualised by CLSM at x400 magnification. Scale bar represents 20 μm. (A) represents an intact monolayer of 16HBE 14o$^-$ cells. (B) An intact monolayer of 16HBE 14o$^-$ cells stimulated with 100 ng/ml HGF. Images are representative of two separate experiments.

Immunostaining for FXIIIA demonstrated that this coagulation factor is expressed by 16HBE 14o$^-$ cells (*figure 3.23 A*) and that HGF stimulates the diffusion of FXIIIA from the cytoplasm to the plasma membrane (*figure 3.23 B*).

3.4.5. PGE_2

Since PGE_2 is the predominant prostanoid produced by the bronchial epithelium and has been implicated in wound repair, its release was investigated in this model of 16HBE 14o$^-$ wound repair.

3.4.5.1. PGE_2 ELISA

Figure 3.24 represents the standard curve for PGE_2. Since the assay relies on competitive binding, the optical density of the standard decreases with increasing concentration of PGE_2.

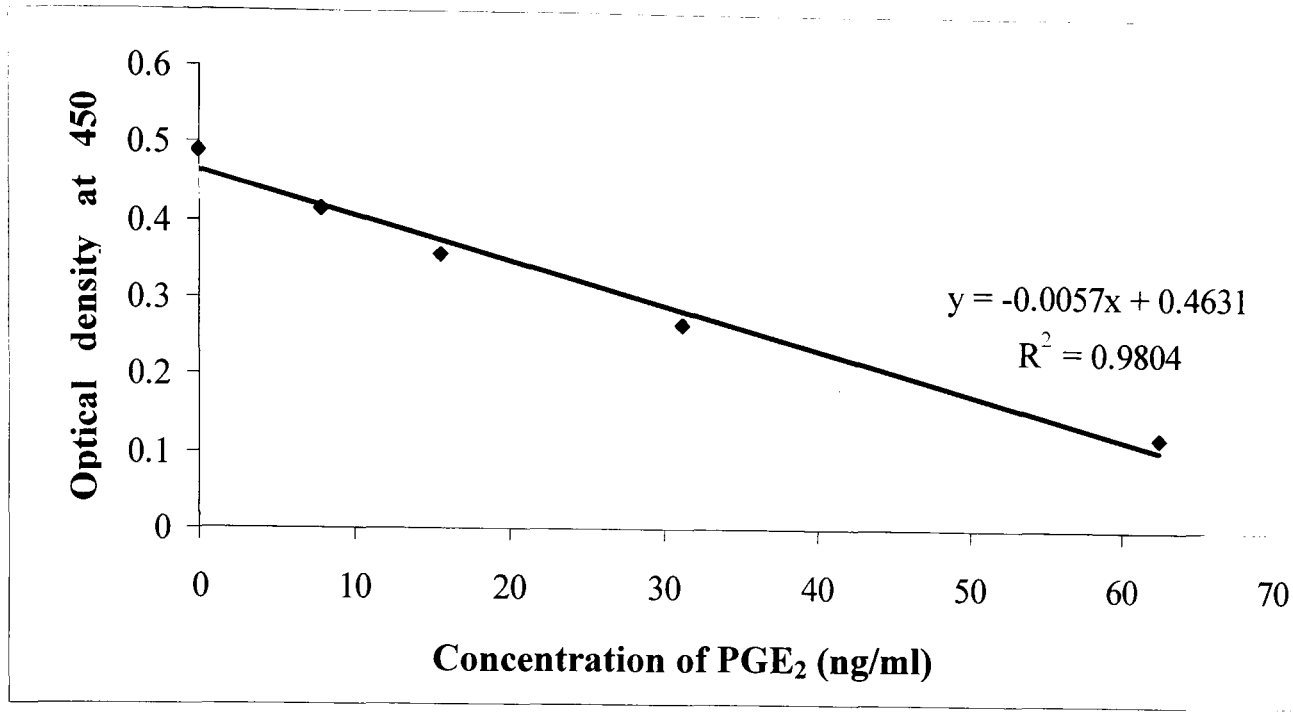

Figure 3.24. Representative graph illustrating the standard curve for PGE_2.

The effect of wounding on the concentration of PGE_2 in cell culture supernatants at 20 minutes was investigated (*table 3.7*).

Degree of wounding at 20mins	Mean optical density (OD) at 405 nm	Mean concentration of PGE_2 (pg/ml)	SEM
C	0.421	9.391	0.822
W8	0.424	8.841	1.577

Table 3.7. Optical density values indicating the effect of wounding on the concentration of PGE_2 in cell culture supernatants of 16HBE 14o$^-$ cells at 20 minutes. C=unwounded cell control and W8=8 wounds. Data represent mean ± SEM (*n=3*).

Similarly, the effect of wounding on the concentration of PGE_2 in cell culture supernatants at 2 hours was investigated (*table 3.8*).

Degree of wounding at 2h	Mean optical density (OD) at 405 nm	Mean concentration of PGE_2 (pg/ml)	SEM
C	0.420	9.526	0.338
W8	0.417	9.792	0.523

Table 3.8. Optical density values indicating the effect of wounding on the concentration of PGE_2 in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. C=unwounded cell control and W8=8 wounds. Data represent mean ± SEM (*n=3*).

The concentration levels of this prostanoid were found to be very low in cell culture supernatants at baseline and in response to wounding at 20 minutes and 2 hours. Furthermore, there was no significant difference between conditions at each of the time-points investigated.

3.4.5.2. Effect of PGE_2 on fibrinogen expression

Previous studies had indicated a positive effect of PGE_2 at concentrations of up to 10 μg/ml on repair of wounded 16HBE 14o$^-$ monolayers (Savla *et al.*, 2001). Therefore, the effect of exogenously added PGE_2 (0-20 μg/ml) on the release of fibrinogen from intact and wounded cell monolayers at 20 minutes and 2 hours was investigated by immunoblot analysis.

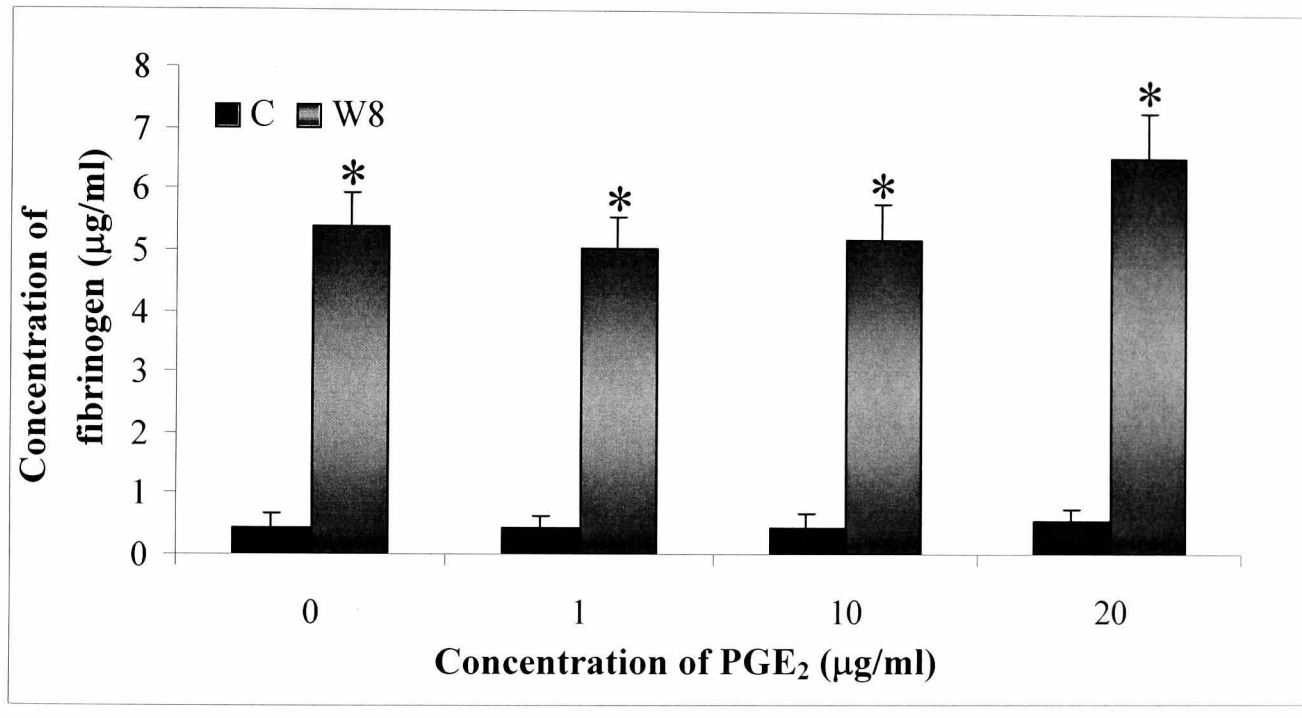

Figure 3.25. Effect of PGE_2 on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells at 20 minutes. C denotes control (unwounded) cells and W8 represents maximum scrape damage. Data represent mean ± SEM (*n=3*). * Indicates $P<0.05$ compared to unwounded cell control.

The concentration of fibrinogen in supernatants of the wounded cell culture control was significantly increased compared to the unwounded cell culture control at 20 minutes (W8=5.38 ± 0.55 compared to C=0.45 ± 0.20). This pattern remained the same with the addition of PGE_2. With respect to the wounded cell cultures, the addition of 20 μg/ml PGE_2 resulted in a slightly higher concentration of fibrinogen in cell culture supernatants compared to the no drug control, however, this effect was insignificant.

Thus, the prostanoid did not induce any significant effects on the levels of fibrinogen in cell culture supernatants in the intact or the wounded cell cultures at 20 minutes.

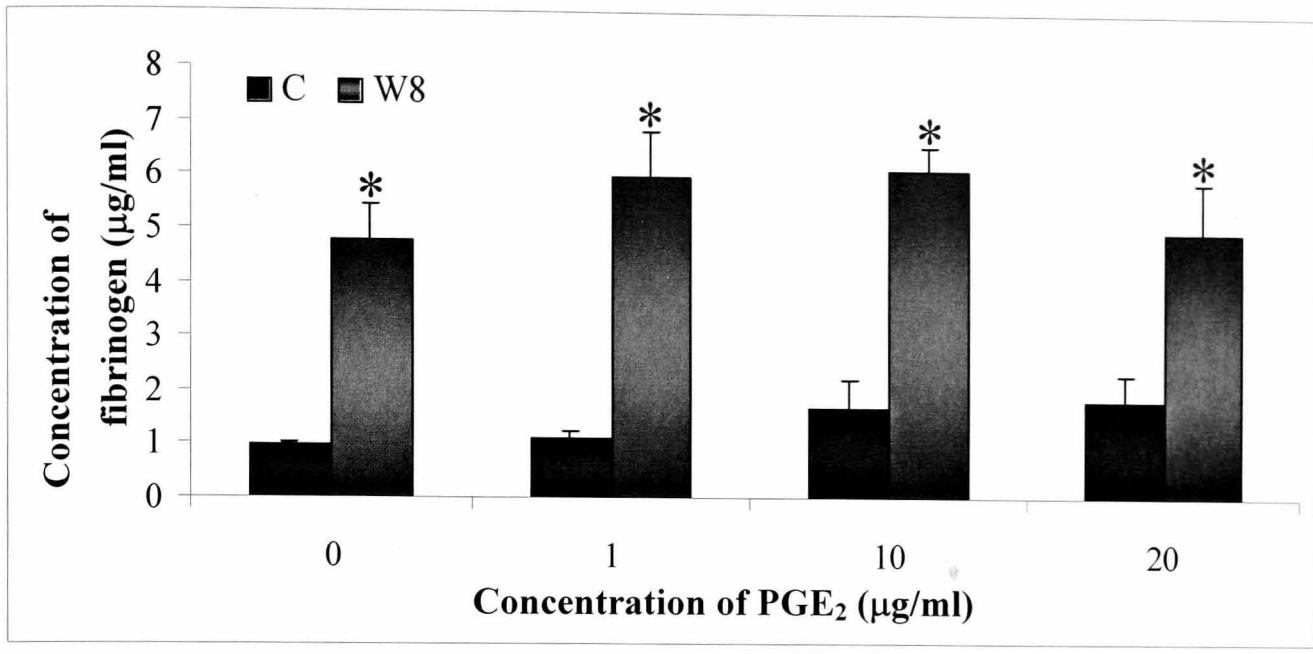

Figure 3.26. Effect of PGE_2 on the concentration of fibrinogen in cell culture supernatants of 16HBE $14o^-$ cells at 2 hours. C denotes control (unwounded) cells and W8 represents maximum scrape damage. Data represent mean ± SEM ($n=3$). * Indicates $P<0.05$ compared to unwounded cell control.

At 2 hours, there was a significant increase in the concentration of fibrinogen released into supernatants of the wounded cell cultures compared to baseline in the absence of PGE_2 (W8=4.78 ± 1.25 µg/ml compared to C=0.96 ± 0.06 µg/ml). With the addition of PGE_2, the difference between fibrinogen concentration in supernatants of baseline and wounded cell cultures remained significant, however, PGE_2 did not affect the release of fibrinogen into supernatants under either condition.

3.4.5.3. Effect of PGE_2 on FXIII concentration

Similarly, the effect of PGE_2 (0-20 µg/ml) on the release of FXIII from intact and wounded cell monolayers at 20 minutes and 2 hours was investigated by immunoblot analysis.

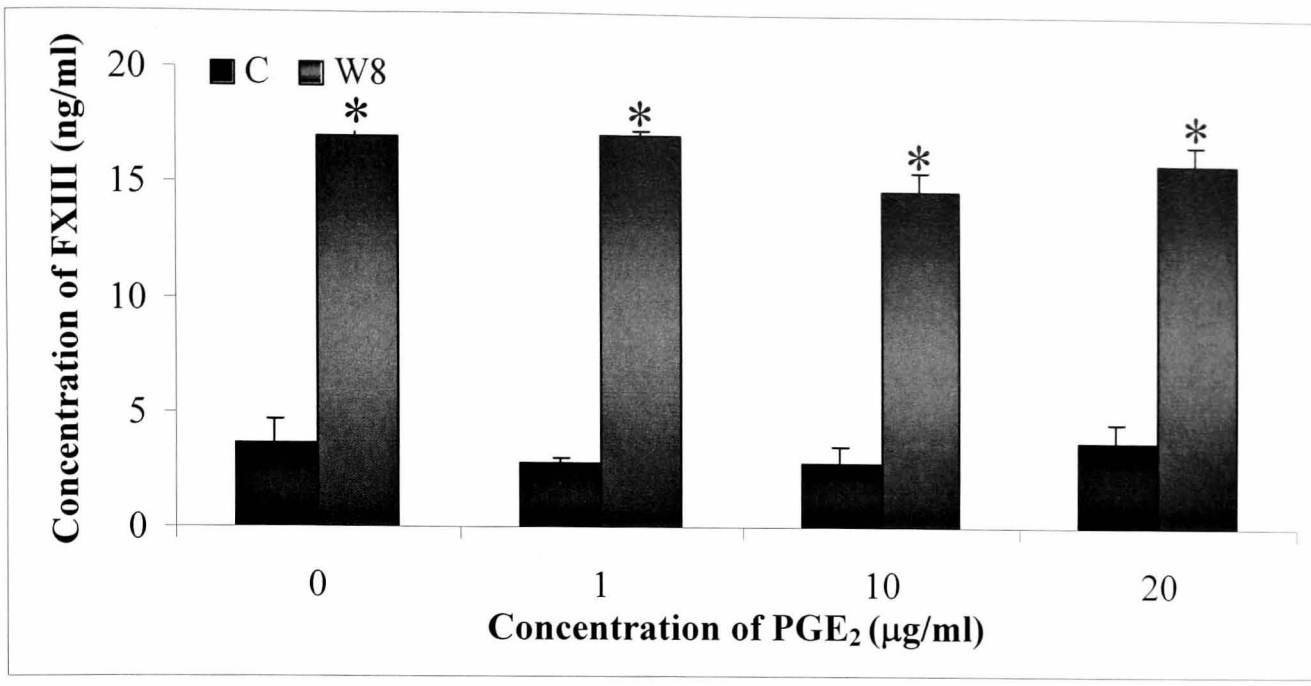

Figure 3.27. Effect of PGE_2 on the concentration of FXIII in cell culture supernatants of 16HBE 14o$^-$ cells at 20 minutes. C denotes control (unwounded) cells and W8 represents maximum scrape damage. Data represent mean ± SEM (*n=3*). * Indicates $P<0.01$ compared to unwounded cell control.

With respect to the control cell cultures, a significantly higher level of FXIII was present in supernatants derived from wounded cells (17.01 ± 0.91 ng/ml) compared to those from unwounded cell monolayers (3.67 ± 0.98 ng/ml) at 20 minutes. The addition of PGE_2 (1-20 µg/ml) had no effect on the concentration of FXIII in supernatants at baseline or in the wounded cell cultures.

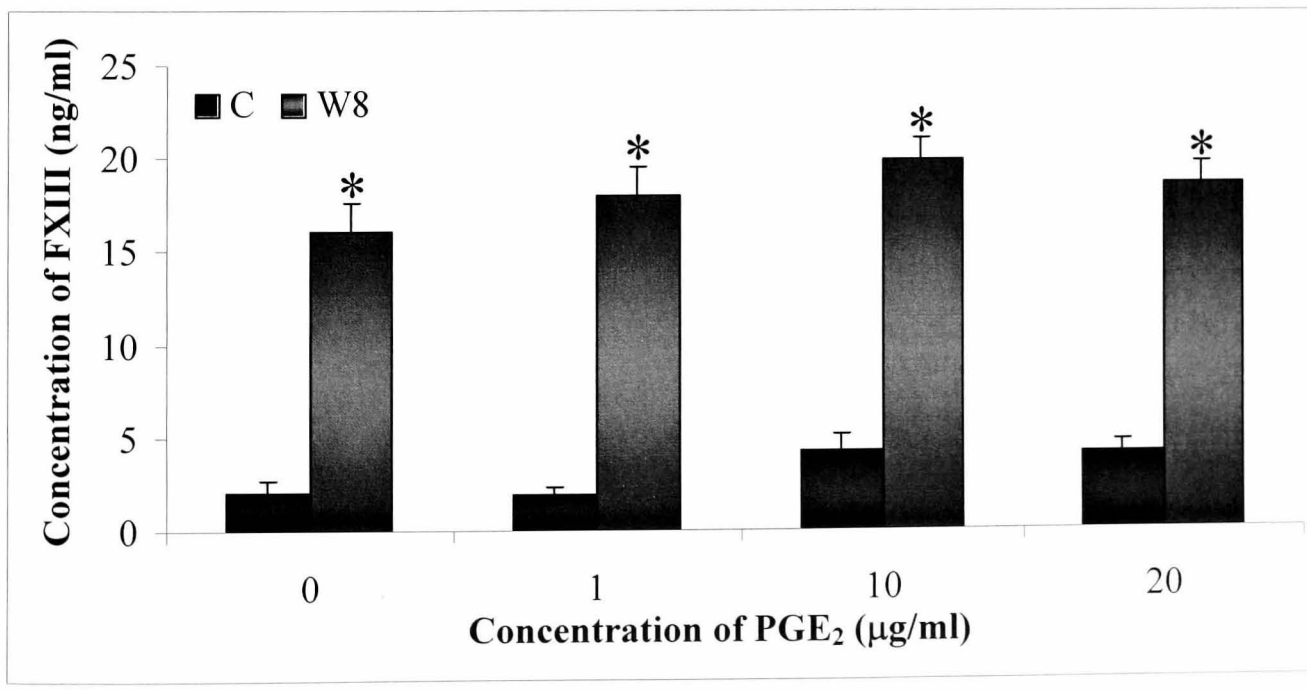

Figure 3.28. Effect of PGE_2 on the concentration of FXIII in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. C denotes control (unwounded) cells and W8 represents maximum scrape damage. Data represent mean ± SEM (*n=3*). * Indicates $P<0.01$ compared to unwounded cell control.

The concentration of FXIII in supernatants derived from wounded cell culture controls was significantly higher (16.05 ± 1.55 ng/ml) than that demonstrated in supernatants from the unwounded cell culture controls (2.04 ± 0.68 ng/ml) at 2 hours. This pattern remained the same following the addition of PGE_2 and the prostanoid had no effect on the concentration of FXIII in supernatants at baseline or the wounded cell cultures.

3.4.5.4. Effect of PGE_2 on D-dimer concentration

Finally, the effect of PGE_2 (0-20 μg/ml) was investigated on the release of D-dimers, the marker of fibrin formation and breakdown, from intact and wounded cell monolayers at 20 minutes and 2 hours by immunoblot analysis.

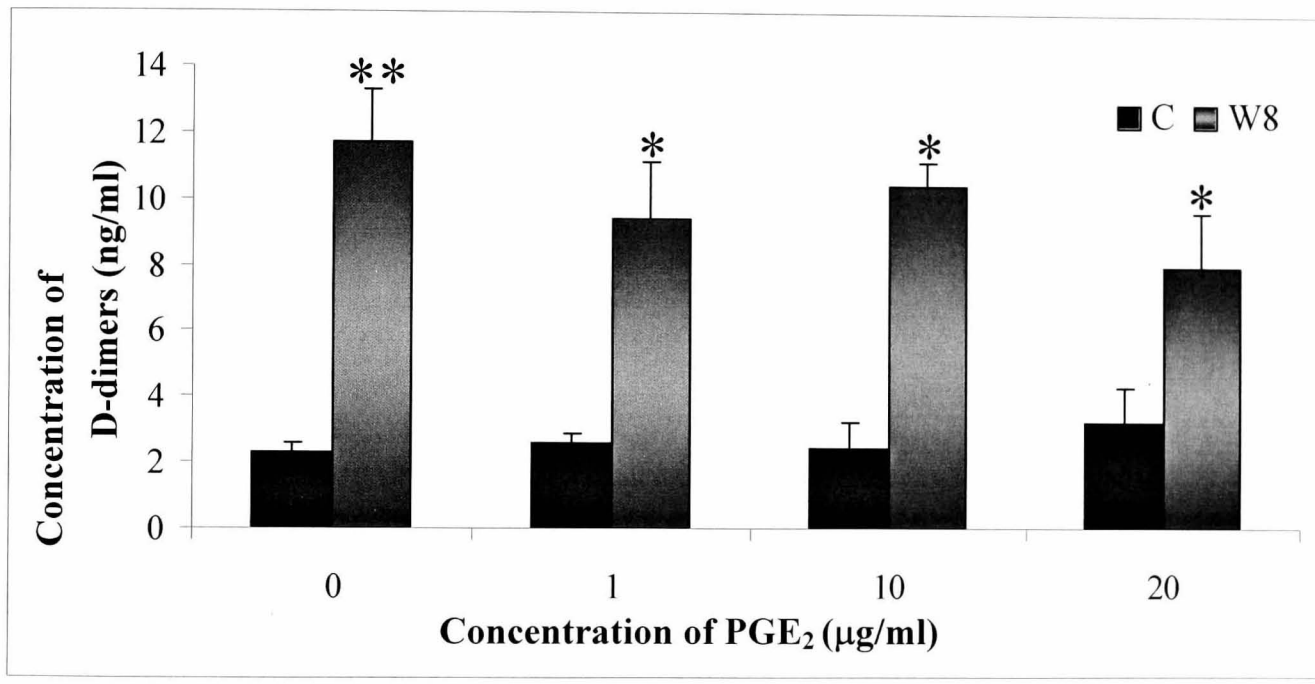

Figure 3.29: Effect of PGE_2 on the concentration of D-dimers in cell culture supernatants of 16HBE 14o$^-$ cells at 20 minutes. C denotes control (unwounded) cells and W8 represents maximum scrape damage. Data represent mean ± SEM (*n=3*). * Indicates P<0.01 and * indicates P<0.001 compared to unwounded cell control.

Analysis of D-dimer concentration in supernatants at 20 minutes revealed that wounding alone stimulated a significantly higher level of D-dimers compared to baseline (W8=11.73 ± 1.53 ng/ml and C=2.27 ± 0.31 ng/ml). This effect remained the same with the addition of PGE_2 and the prostanoid had no effect on the levels of D-dimers in supernatants at baseline or those of cells subjected to wounding.

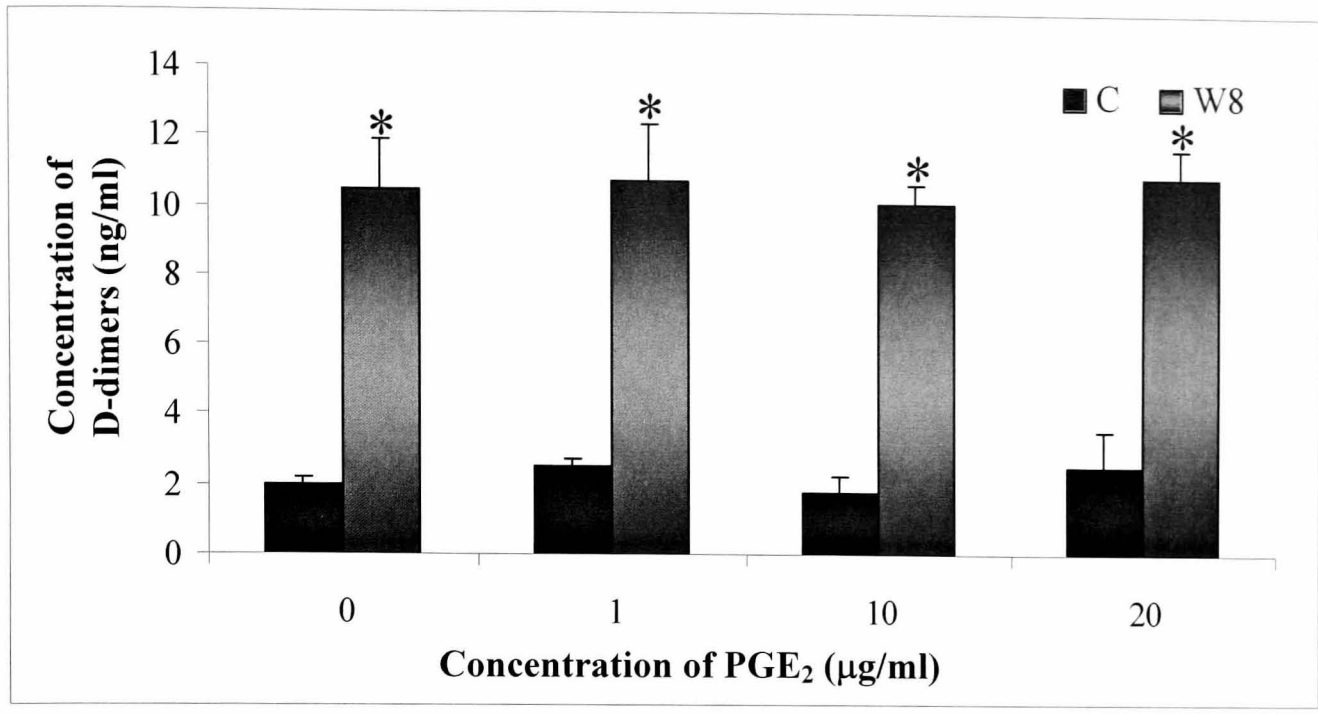

Figure 3.30. Effect of PGE_2 on the concentration of D-dimers in cell culture supernatants of 16HBE cells at 2 hours. C denotes control (unwounded) cells and W8 represents maximum scrape damage. Data represent mean ± SEM (*n=3*). * Indicates $P<0.01$ compared to unwounded cell control.

Similarly, at 2 hours, supernatants of wounded cell cultures displayed a significantly higher level of D-dimers compared to that seen at baseline (W8=10.46 ± 1.45 ng/ml and C=2.00 ± 0.18 ng/ml). This effect was unaltered by PGE_2 and the prostanoid did not induce any effect on the D-dimer concentration in supernatants at baseline or of the wounded cell cultures.

3.4.6. IL-8

IL-8, an inflammatory cytokine abundantly expressed in human bronchial epithelial cells, was measured in intact and wounded 16HBE $14o^-$ cell culture supernatants, as described in *section 3.3.8*. *Figure 3.31* illustrates a typical standard curve for the detection of IL-8 in cell culture supernatants. The assay was highly sensitive, with a detection limit of 1 pg/ml, therefore samples were diluted 1 in 10 prior to assay.

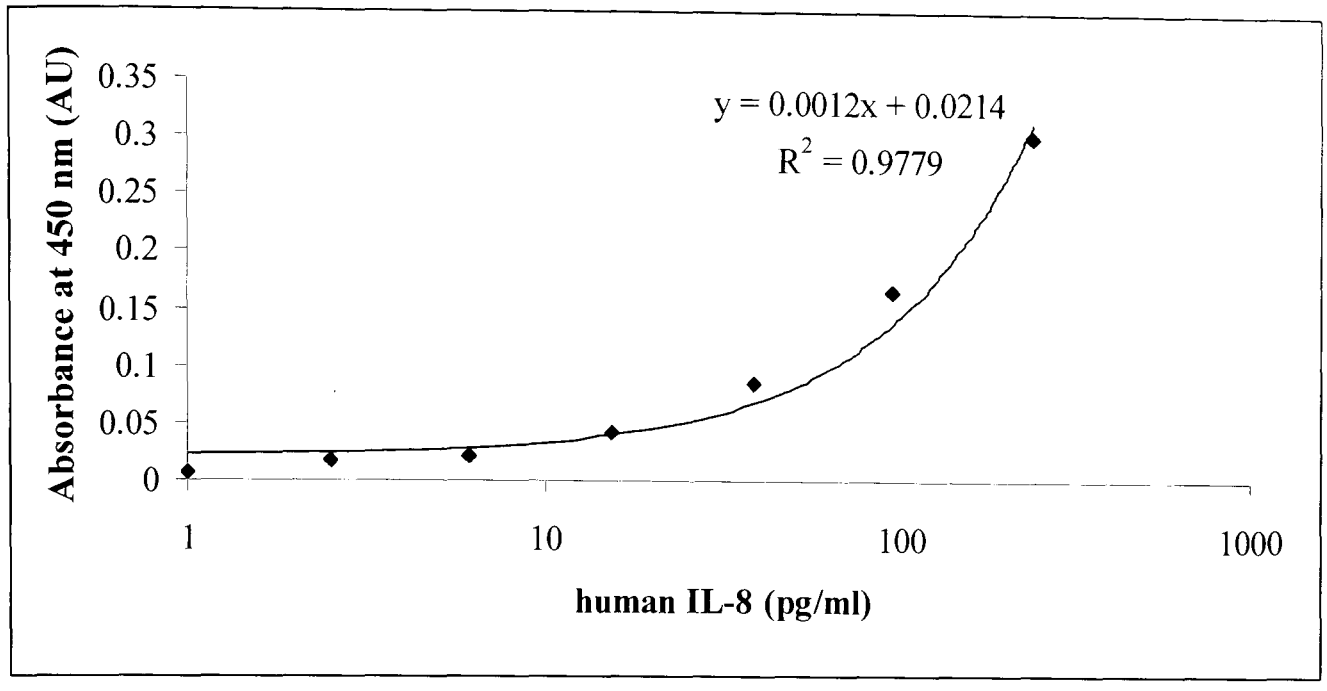

Figure 3.31. Representative graph illustrating the standard curve for IL-8.

Figure 3.32 represents the release of IL-8 into cell culture supernatants up to 12 hours *post*-wounding, determined by IL-8 ELISA.

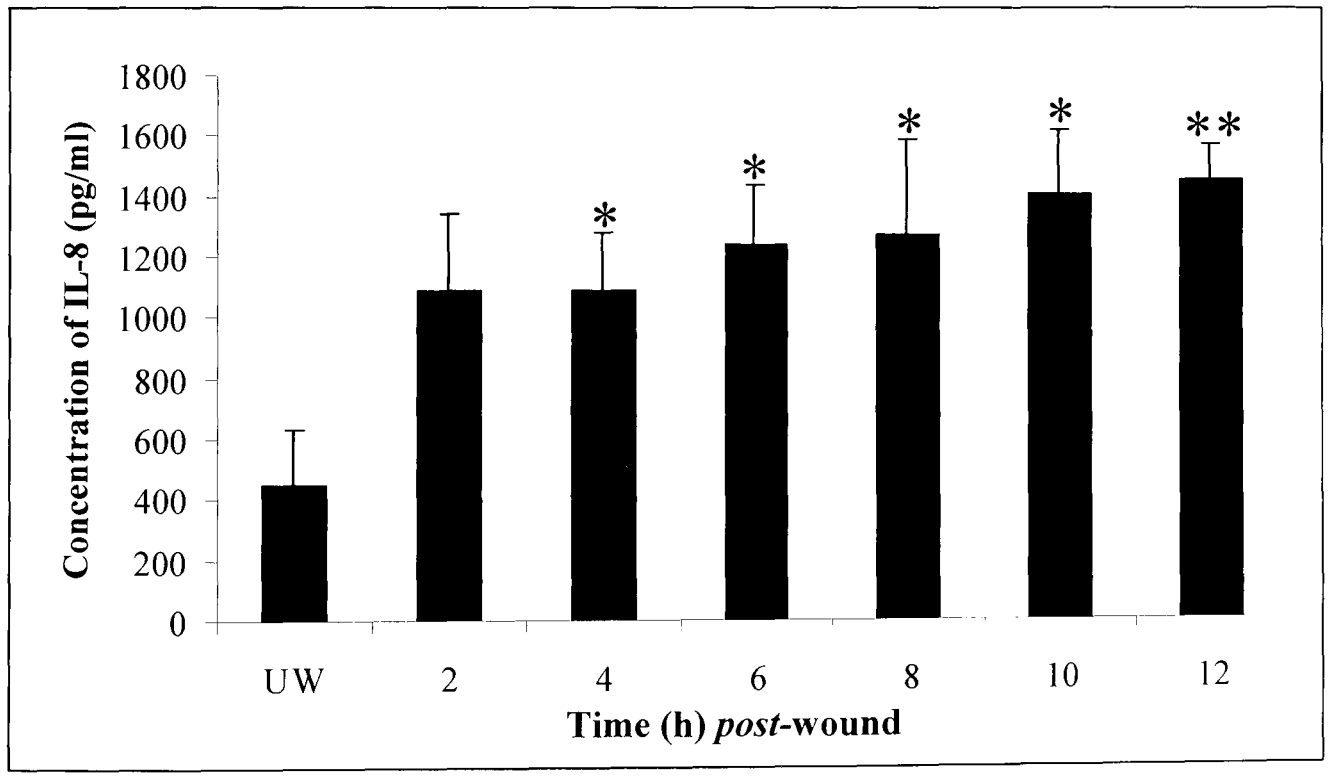

Figure 3.32. Effect of time *post*-wounding on the concentration of IL-8 in cell culture supernatants of 16HBE 14o$^-$ cells. UW= unwounded cells. 12 hours cell culture supernatant. Data represent mean ± SEM (n=5). * Indicates P<0.05 and ** indicates P<0.005 compared to unwounded cell control.

Analysis of cell culture supernatants for IL-8 revealed that there was a significant increase with time 4 hours *post*-wounding (1093.3 ± 188.9) and this was maintained up to 12 hours (1450.8 ± 120.5) compared to the unwounded cell control (449.2 ± 186.3).

3.4.7. Neutrophil elastase

Neutrophil elastase has previously been reported to enhance IL-8 expression (Devaney *et al.*, 2003) and increase PGE_2 production (Perng *et al.*, 2003) in bronchial epithelial cells and might therefore enhance coagulation factor expression in the current model. Activation of PAR-2 was shown to enhance the release of coagulation factors from 16HBE $14o^-$ cells in *Chapter 5,* however, neutrophil elastase has been reported to inactivate PAR-2 (Dulon *et al.*, 2003); therefore neutrophil elastase might inhibit the release of coagulation factors in the current model. Neutrophil elastase is also known to cleave coagulation factors (Anderssen *et al.*, 1993; Turkington, 1991). Therefore, the effect of neutrophil elastase on coagulation factor expression was investigated.

3.4.7.1. Effect of neutrophil elastase on fibrinogen concentration

The effect of neutrophil elastase was investigated on the release of fibrinogen into cell culture supernatants 20 minutes and 2 hours *post*-wounding by immunoblot analysis. A concentration range of 0-0.5 U/ml was used since 50 nM neutrophil elastase (corresponding to approximately 0.5 U/ml) was previously shown to upregulate IL-8 (Devaney *et al.*, 2003).

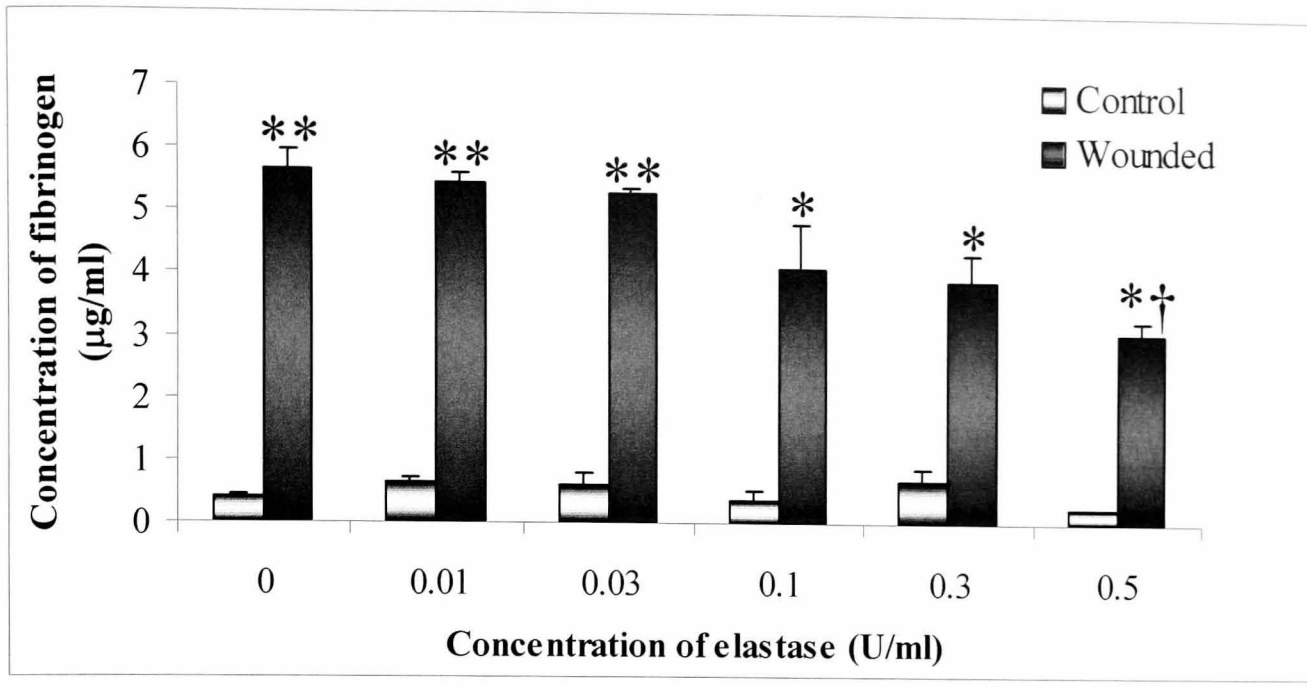

Figure 3.33. Effect of neutrophil elastase on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells at 20 minutes. Control bars denote unwounded cells and wounded bars represent maximum scrape damage, W8. Data represent mean ± SEM (*n=3*). * Indicates P<0.01 and ** indicates P<0.001 compared to control (unwounded) cells at each concentration of elastase. † Indicates P<0.05 compared to the no elastase control in the wounded cells.

With respect to the control cell cultures, wounding alone stimulated a significant increase in the concentration of fibrinogen in supernatants (5.66 ± 0.31 µg/ml) compared to baseline (0.41 ± 0.01 µg/ml) at 20 minutes. This pattern was maintained up to 0.5 U/ml elastase. However, the addition of 0.5 U/ml elastase induced a significant decrease in the concentration of fibrinogen in supernatants of the wounded cell cultures (3.05 ± 0.21 µg/ml) compared with the no drug control (5.66 ± 0.31 µg/ml). Elastase had no effect on the concentration of fibrinogen in supernatants of the unwounded cell cultures.

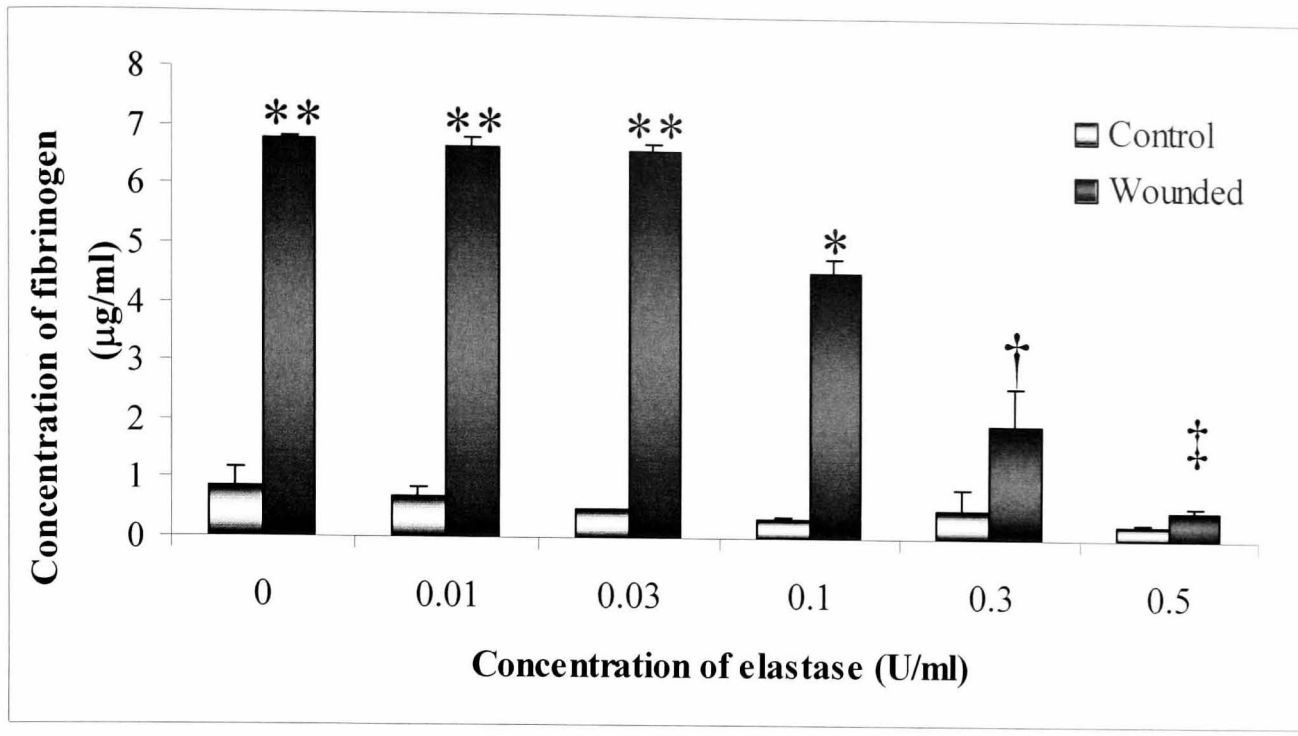

Figure 3.34. Effect of elastase on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. Control bars denote unwounded cells and wounded bars represent maximum scrape damage, W8. Data represent mean ± SEM ($n=3$). * Indicates $P<0.01$ and ** indicates $P<0.001$ compared to control (unwounded) cells at each concentration of elastase. † Indicates $P<0.05$ and ‡ indicates $P<0.001$ compared to the no elastase control in the wounded cells.

With respect to the control cell cultures, the concentration of fibrinogen in supernatants derived from wounded cell monolayers (6.77 ± 0.04 µg/ml) was significantly higher at 2 hours compared to that seen at baseline (0.85 ± 0.33 µg/ml). This pattern was maintained following addition of up to 0.1 U/ml elastase. Supernatants of wounded cell cultures subjected to 0.3 U/ml elastase displayed a significantly lower concentration of fibrinogen (1.96 ± 0.64 µg/ml) compared to the no drug control (6.77 ± 0.04 µg/ml). Supernatant concentration of fibrinogen was further diminished to 0.49 ± 0.09 µg/ml with the addition of 0.5 U/ml elastase in the wounded cell cultures. The concentration of fibrinogen in the unwounded cell culture supernatants was unaffected by elastase.

3.4.7.2. Effect of neutrophil elastase on FXIII concentration

The effect of neutrophil elastase (0-0.5 U/ml) on the release of FXIII into cell culture supernatants at 20 minutes and 2 hours was investigated by immunoblot analysis.

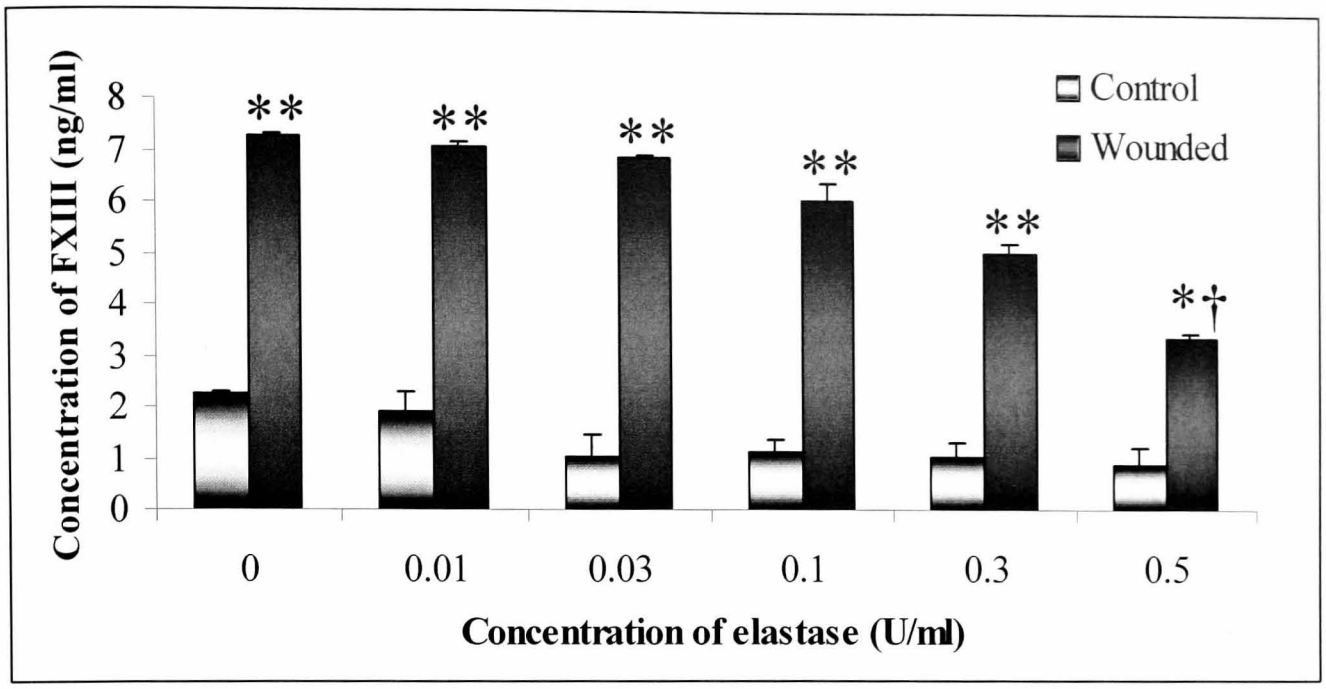

Figure 3.35. Effect of elastase on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells at 20 minutes. Control bars denote unwounded cells and wounded bars represent maximum scrape damage, W8. Data represent mean ± SEM (*n=3*). * Indicates P<0.01 and ** indicates P<0.001 compared to control (unwounded) cells at each concentration of elastase. † Indicates P<0.05 compared to no drug control in the wounded cells.

Analysis of cell culture supernatants at 20 minutes revealed that in the absence of elastase, the wounded cell cultures demonstrated a significantly higher concentration of FXIIIA (7.26 ± 0.05 ng/ml) compared to baseline (2.25 ± 0.04 ng/ml). This effect was evident with addition of each concentration of elastase up to 0.5 U/ml, however, in the wounded cultures subjected to 0.5 U/ml elastase, the concentration of FXIIIA (3.38 ± 0.09 ng/ml) was significantly lower than that seen in the wounded cultures of the no drug control (7.26 ± 0.05 ng/ml). There was no significant effect of elastase on the concentration of FXIIIA in supernatants of the unwounded cells.

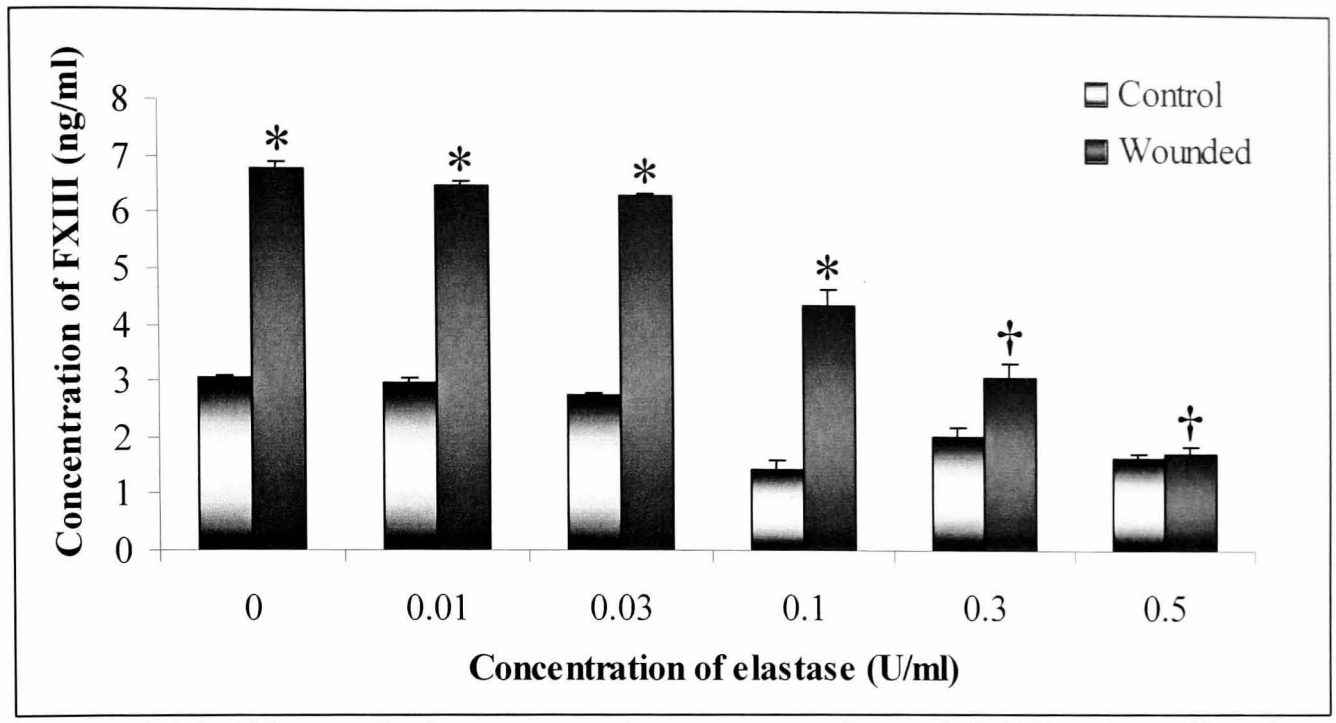

Figure 3.36. Effect of elastase on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells at 2 hours. Control bars denote unwounded cells and wounded bars represent maximum scrape damage, W8. Data represent mean ± SEM (*n=3*). * Indicates P<0.05 compared to control cells at each concentration of elastase. † Indicates P<0.05 compared to the no elastase control in the wounded cells.

With reference to the no drug control, the concentration of FXIIIA in supernatants of the wounded cell cultures (6.77 ± 0.11 ng/ml) was significantly increased with respect to that seen at baseline (3.03 ± 0.05 ng/ml). This pattern was maintained with the addition of elastase up to 0.1 U/ml. There was a significant decrease in the concentration of FXIIIA following the addition of 0.3 U/ml elastase (3.10 ± 0.25 ng/ml) compared to the wounded cell cultures of the no elastase control (6.77 ± 0.11 ng/ml). Furthermore, the concentration of FXIIIA in cell culture supernatants of the wounded cells was further decreased to 1.76 ± 0.13 ng/ml following the addition of 0.5 U/ml elastase compared to the wounded cell cultures of the no drug control. Finally, there was no effect of elastase on the concentration of FXIIIA in the unwounded cell cultures.

3.5. Summary of results

16HBE 14o$^-$ cells demonstrated constitutive expression and activity of TF. A significant increase in the release of fibrinogen, FXIIIA and D-dimers from 16HBE 14o$^-$ cells in response to wounding was demonstrated in the absence of plasma. Moreover, 16HBE 14o$^-$ cells demonstrated expression of FXIIIA by immunohistochemistry. Analysis of supernatant concentration levels of EGF, KGF, TGF-β1 by ELISA indicated that expression of these growth factors was negligible and expression of HGF was not

determined by either ELISA or immunohistochemistry. With respect to the analysis of inflammatory mediators, supernatant concentrations of PGE_2 were determined to be low and levels were unaffected by wounding. Subsequently, the prostanoid had no effect on the concentrations of fibrinogen, FXIIIA or D-dimers in neither the unwounded or wounded 16HBE 14o$^-$ cell cultures. A significant increase in the release of IL-8 was demonstrated in 16HBE 14o$^-$ cell culture supernatants at 4 hours *post*-wounding. Finally, elastase was demonstrated to induce a significant decrease in the release of fibrinogen and FXIIIA in cell culture supernatants of wounded 16HBE14o$^-$ cell monolayers.

3.6. Discussion

The 16HBE 14o$^-$ cell line was chosen for its human origin, physiological barrier properties and convenience of culture. The 16HBE 14o$^-$ cell line describes an SV40 large T-antigen transformed epithelial cell line derived from human bronchial epithelium (Cozens *et al.*, 1994). The well established cell line retains many features of bronchial epithelial cells *in vivo*, including the 'cobblestone' appearance and possession of tight junctions. The cell line is known to mimic the barrier properties that are displayed in the airways *in vivo*. Monolayers of 16HBE 14o$^-$ cells have been shown to generate transepithelial resistance, as measured by Ussing chambers that is comparable to that reported for airway epithelia (Bhat *et al.*, 1993; Rojanasakul *et al.*, 1992).

This *in vitro* model is highly reductive in nature, since it solely consists of a monolayer of undifferentiated 16HBE 14o$^-$ cells and rules out the contribution of the underlying mesenchyme, a source of growth factors including EGF, KGF, TGF-β and HGF, all of which are reported to play a role in wound repair; plasma, a source of coagulation factors and sensory neuropeptides such as vasoactive intestinal peptide (VIP), which is mainly produced by non-adrenergic and non-cholinergic nerve fibres and has recently been shown to enhance wound repair and proliferation of bronchial epithelial cells *via* VIP receptors (Guan *et al.*, 2006). The 16HBE 14o$^-$ cells were grown in submerged cultures and remained undifferentiated as a consequence. Cells were cultured on bare plastic to rule out the effect of the extracellular matrix and individual components such as laminin and collagen type IV, which are known to play a role in cell migration (Rickard *et al.*, 1993). One of the features of the inflammatory process in asthma is the

presence of increased plasma leakage due to plasma extravasation from the blood-airway lumen barrier; however, plasma exudation following allergen challenge does not occur in normal subjects (Nocker *et al.*, 1999). To rule out the effect of plasma-derived proteins, 16HBE 14o$^-$ cells were quiesced overnight in serum-free medium and prior to each experiment, the serum-free medium was refreshed and the experiments were performed in the same medium. Although this model may be considered far removed from the conditions demonstrated *in vivo*, it allows the contribution of the bronchial epithelium alone to be investigated, in the absence of plasma proteins.

Preliminary results from western blotting indicated both high molecular weight forms of coagulation factors as well as proteolysed fragments. For example, FVII was demonstrated as part of the TF:FVII complex as described in *Chapter 7* and two low molecular weight fragments of 32 and 20 kDa, indicative of tryptic digestion (Kazama *et al.*, 1993). Trypsin is a product of injured epithelial cells (Reed *et al.*, 2004) and may account for the proteolysis. Proteolysis clearly occurred during cell culture, as adding a cocktail of protease inhibitors (including: 500 μM AEBSF, hydrochloride; 150 nM aprotinin; 1 μM E-64; 0.5 mM EDTA, disodium and 1μM leupeptin, hemisulphate: protease inhibitor cocktail set 1; supplied by Calbiochem®) to harvested cells did not prevent its occurrence. With the exception of FXIII, the coagulation factors are themselves proteases; therefore protease inhibitors could not be added to cell cultures. Thus, immunoblotting was considered the most appropriate technique for protein analysis since it detects the sum of both high molecular weight complexes and tryptic fragments.

It is well established that TF is an integral membrane glycoprotein that is constitutively expressed in cells, at sites where it may play a protective role in wound repair (Osterud, 1997). TF is the principal initiator of coagulation *in vivo* as a consequence of its binding to FVII and the formation of a complex that activates FX (Nemerson, 1966). In the airways, TF is expressed at epithelial barriers which are physically separated from the circulating blood. The specific localisation of TF provides a 'hemostatic envelope', such that only vascular injury may initiate the activation of coagulation (Martin *et al.*, 1998). 16HBE 14o$^-$ cells demonstrated positive, specific immunostaining for TF by confocal laser scanning microscopy. TF immunostaining was constitutive and was localised to the periphery of cells within the 16HBE 14o$^-$ monolayer. The expression of TF by

16HBE 14o$^-$ cells in the current model supports the results of a previous investigation which showed that TF was expressed and active on primary normal human bronchial epithelial (NHBE) cells in culture (Keller *et al.*, 2001). Analysis of TF activity of 16HBE 14o$^-$ cells demonstrated that TF is constitutively active. Wounding induced a small but non-significant increase in TF activity compared to baseline. It is likely that the small increase relates to the upregulation of TF in the small proportion of cells at the wound edge as indicated by immunohistochemistry images in *section 3.4.1.1.*

The data presented in this chapter provide evidence that the bronchial epithelium is a source of coagulation factors that are released in response to injury. Following wounding, cell culture supernatants demonstrated a significant increase in the concentrations of fibrinogen and FXIIIA at 2 hours, indicating release of these factors from 16HBE 14o$^-$ cells dependent on the extent of wounding. The functional significance of these observations is described in *Chapter 4*.

The liver is the primary source of plasma fibrinogen *in vivo*, and hepatic fibrinogen is upregulated as part of the innate immune response to inflammation (Baumann *et al.*, 1994; Otto *et al.*, 1987). Previous studies have demonstrated expression and synthesis of fibrinogen in extraheptic, alveolar epithelial (A549) cells *in vitro*, the production of which was found to be regulated by interleukin (IL)-6 and dexamethasone and was independent of cleavage by thrombin. The fibrinogen was demonstrated to be released from alveolar cells and incorporated into a fibrinogen matrix, independently of conversion to fibrin (Guadiz *et al.*, 1997; Haidaris, 1997). The data presented here confirm that a bronchial epithelial cell line also expresses fibrinogen and that its abundance is increased in response to injury. The expression and release of fibrinogen from 16HBE 14o$^-$ cells, whereby mechanical wounding is the stimulus for its upregulation is a novel finding. Cell culture supernatants at baseline contained 0.35 μg/ml fibrinogen, which is comparable to 0.64 μg/ml fibrinogen reported in supernatants derived from a human hepatoma cell line at baseline (Fair *et al.*, 1983) and 0.15 μg/ml fibrinogen reported in BAL fluid from patients with mild, stable asthma (Pizzichini *et al.*, 1998).

Plasma FXIII is a zymogen of tetrameric structure (A_2B_2) consisting of two potentially active A subunits (FXIIIA) and two inhibitory/carrier B subunits (FXIIIB) (Muszbek *et*

al., 1999). The A-subunit contains the active site of the enzyme and is expressed by platelets, monocytes, macrophages (Henriksson *et al.*, 1985; Kradin *et al.*, 1987; Muszbek *et al.*, 1999) and hepatocytes (Adany *et al.*, 1996). The B-subunit is synthesised in the liver, and is secreted as a monomer that binds free FXIIIA in plasma (Nagy *et al.*, 1988). The main function of the B-subunit is the stabilisation and transport of the hydrophobic A-subunit in the aqueous environment of human plasma (Ariens *et al.*, 2002). An antibody to FXIIIA was used in this model, since this is the cell-associated form. The data in this chapter demonstrate that FXIIIA is expressed in the 16HBE 14o$^-$ cell line and its expression is increased in response to injury. This represents a highly novel observation since bronchial epithelial cells have not previously been shown to express FXIIIA. A study involving the analysis of BAL fluid from healthy children compared to those with bronchoalveolar inflammation demonstrated a low amount of FXIIIA present in BAL fluid from non-diseased subjects that derived from alveolar macrophages and not from plasma; and FXIIIB was not present. Subjects with bronchoalveolar inflammation displayed elevated concentrations of FXIIIA in the BAL fluid with the additional presence of $FXIIIA_2B_2$, indicating a sign of increased capillary permeability and plasma exudation as a marker of inflammation (Katona *et al.*, 2005). Interestingly, cellular FXIII, which lacks the inhibitory B subunit, does not require the release of the activation peptide for its activation, since Ca^{2+} present in the bronchoalveolar lining fluid is sufficient to convert this zymogen into an active enzyme (Polgar *et al.*, 1990). In order to determine the activity of the FXIIIA released into cell culture supernatants in this model, a FXIII activity assay would need to be performed. The assay was not employed since it was deemed to be both too complicated and time-consuming to optimise.

Bronchial epithelial repair is reported to rely on the formation of a provisional fibrin matrix (Erjefalt *et al.*, 1994). TF, fibrinogen and FXIII are all mediators of fibrinogenesis, therefore, detection of these factors in this model and an increase in their expression in response to wounding suggests that these factors are available for fibrin formation.

D-dimers represent the breakdown products of either a fibrin or fibrinogen clot that has been stabilised by FXIIIA cross-linking. Fibrin provides a matrix for wound repair but is not a permanent structure. In order to prevent fibrosis, it must be degraded into

soluble D-dimer fragments once it has performed its duty (Sidelmann *et al.*, 2000). The fibrinolytic pathway is described in *section 3.1.6*. In brief, the fibrin clot is degraded by plasmin, the fibrinolytic enzyme, which is generated by the proteolytic cleavage of plasminogen, which circulates in the blood. Activation of plasminogen may either occur directly by t-PA, which is produced and released from vascular endothelial cells; by scu-PA, a part of the intrinsic system, which can be converted into active u-PA by various proteases; or by the FXII-dependent pathway. D-dimers are a marker of both fibrinolysis and fibrinogenesis; therefore the increased expression of these fragments in cell culture supernatants provides evidence that cross-linked fibrin was generated in response to wounding and then degraded, and that its formation was dependent on the extent of cell damage.

In addition to the expression of TF, FVII and fibrinogen that was demonstrated by 16HBE 14o$^-$ cells in the current study, the generation of both FXIII and D-dimers suggests that there is thrombin expression and activity in the current 16HBE 14o$^-$ cell model. Subsequently, this implies that coagulation factors Xa and Va and prothrombin are present in the current model and thus synthesised by 16HBE 14o$^-$ cells in order for the generation of thrombin. Furthermore, the expression of D-dimers in this model suggests that plasmin must be present. This is conceivable since alveolar epithelial cells have been shown to express u-PA and have the capacity to promote fibrinolysis following fibrin deposition (Marshall *et al.*, 1992).

LDH measurements of cell culture supernatants demonstrated a small, insignificant increase in release of LDH in response to wounding. The increase in LDH in the supernatants correlated with the slight decline of LDH in the lysates of the 16HBE 14o$^-$ cells with the extent of wounding. The levels of LDH in supernatants of unwounded cell culture controls represent 1% of the total and indicate that there is a limited amount of cell death under the current culture conditions. However, wounding did not induce a significant increase in the proportion of LDH in the supernatants. This signifies that the release of coagulation factors into cell culture supernatants was predominantly from intact cells within the wounded cell monolayer and not from cells that were damaged and lysed by mechanical wounding. Furthermore, it is reported that bronchial epithelial cells that are shed from asthmatic subjects remain viable (Montefort *et al.*, 1992).

Mechanical wounding destroys the tight junctions that maintain the integrity of the bronchial epithelium. Tight junctions comprise at least four types of transmembrane proteins, including occludin, claudins, junctional adhesion molecules and cadherins, and a number of cytoplasmic peripheral proteins, such as the PDZ proteins, which include the ZO family. Whereas the transmembrane proteins mediate cell-cell adhesion (Tsukita *et al.*, 1999), the cytosolic tight junction plaque contains various types of proteins that link tight junction transmembrane proteins to the underlying cytoskeleton (Balda *et al.*, 2000; Itoh *et al.*, 1999; Wittchen *et al.*, 1999). These adapter proteins also recruit regulatory proteins, such as protein kinases, phosphatases, small GTPases and transcription factors, to the tight junction. As a result, structural proteins such as actin and spectrin; and regulatory proteins such as actin-binding proteins, GTPases and kinases are juxtaposed with transmembrane proteins. This protein scaffolding facilitates the assembly of highly ordered structures, such as junctional complexes or signalling patches, that regulate epithelial cell polarity, proliferation and differentiation. Epithelial permeability is tightly regulated by intracellular messengers (Lawrence *et al.*, 2002). A number of signalling pathways have been implicated in tight junction biogenesis; however, the precise molecular mechanisms are not fully understood. Since 16HBE $14o^{-}$ cells are known to form confluent monolayers and express various tight junction proteins such as occludin and ZO-1, it would therefore be of interest to further investigate a role for these proteins in wound repair by use of neutralising antibodies to these proteins in order to disrupt the tight junctions. For example, a mouse monoclonal E-cadherin blocking antibody was used to demonstrate that E-cadherin promotes EGFR-mediated cell differentiation and MUC5AC mucin expression in cultured human airway epithelial cells (Kim *et al.*, 2005).

TF was demonstrated to be constitutively expressed and constitutively active in this model; and its expression was increased at the wound edge. The presence of D-dimers in the cell culture supernatants suggests that the binding of FVII with TF is likely, since these fragments are indicative of fibrin formation and subsequent degradation. This was confirmed by preliminary experiments in *Chapter 7*, which demonstrated the formation of a TF:FVII complex. The rapid formation of D-dimers indicates the release of pre-formed coagulation factors. This is supported by data in *Chapter 6* which demonstrates that wounding initiates a significant increase in TF and FVII mRNA over a longer time course. The formation of fibrin represents the final step within the coagulation cascade.

In order to initiate coagulation, TF expressed by the epithelium combines with FVII, which when activated, activates FX. The main source of FVII and FX is considered to be the liver (Greenberg *et al.*, 1995; Hung *et al.*, 1996). In addition, FVII is synthesised by macrophages, smooth muscle cells, fibroblasts and keratinocytes (Wilcox *et al.*, 2003) and FX is expressed by alveolar macrophages (Osterud *et al.*, 1980). However, both FVII and FX have previously been shown to be expressed by 16HBE 14o$^-$ cells (PhD thesis; Perrio, 2006) and these coagulation factors have been demonstrated in 16HBE 14o$^-$ cells at the mRNA level in *Chapters 6 and 7*.

A number of growth factors have been previously reported to play a role in epithelial repair, therefore their expression in this model was investigated. The concentration of EGF in cell culture supernatants, as determined by ELISA, was 0.26 pg/ml 2 hours *post*-wounding. Its expression did not change with time *post*-wounding and was not significantly different to the levels detected at baseline. The levels of EGF detected in this model may be considered too low to stimulate migration compared to a study which demonstrated that 1 ng/ml EGF significantly enhanced wound repair in a model of undifferentiated NHBE cells (Wadsworth *et al.*, 2006). EGF at 10 ng/ml was demonstrated to stimulate the migration of 16HBE 14o$^-$ cells (Puddicombe *et al.*, 2000). EGF is a potent mitogen that is considered to be an important regulator of epithelial restitution due to its ability to stimulate cell migration, proliferation, differentiation and survival (Puddicombe *et al.*, 2000). Weak expression of EGF has been demonstrated in the bronchial epithelium (Amishima *et al.*, 1998). Interestingly, EGFR is reported to be widely expressed by the epithelium. However, its pattern of expression is restricted given that it is expressed basolaterally and is therefore only exposed to EGFR ligands in response to injury. Puddicombe *et al.* demonstrated that following injury of 16HBE 14o$^-$ cells, the EGFR was rapidly activated and that this was likely to occur by the binding of either TGF-α or amphiregulin, which are both expressed by 16HBE 14o$^-$ cells. Thus, the fact that EGF was not found to be abundant in cell culture supernatants and that its expression was unaffected by wounding does not rule out a role for EGFR in wound repair. It is conceivable that EGFR expressed by epithelial cells respond to either TGF-α or amphiregulin released from epithelial cells themselves or EGF released from underlying mesenchymal cells, such as fibroblasts to mediate repair. EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, and epiregulin each have the capacity to bind to the

EGFR and are all regulators of bronchial epithelial restitution (Puddicombe *et al.*, 2000).

KGF is recognised as a key growth factor for the bronchial epithelium. It was first isolated in a lung fibroblast line (Rubin *et al.*, 1989). Within the mesenchyme, KGF is predominantly produced by fibroblasts and smooth muscle; however, unlike other growth factors, KGF displays epithelial specificity since its receptor (KGFR) is expressed only in epithelial cells and therefore only acts in a paracrine manner (Finch *et al.*, 1989). Specifically, KGF targets alveolar epithelial cells *in vitro* (Panos *et al.*, 1993) and *in vivo* (Ulich *et al.*, 1994), acting as a potent mitogen. In addition to its role in lung development and inflammation, KGF plays an important role in wound repair (Ware *et al.*, 2002). Galiacy *et al.* (2003) demonstrated an increase in the rate of epithelial repair compared to unstimulated controls by the presence of 100 ng/ml KGF in a model of primary alveolar epithelial cells. However, the concentration of KGF in the cell culture supernatants of 16HBE 14o$^-$ cells in the current model was 13.9 pg/ml at baseline and is therefore considerably lower than the levels of KGF required to facilitate epithelial repair. Furthermore, its expression was unaffected by mechanical wounding. However, the fact that only low levels were detected does not rule out a role for KGF. *In vivo,* it is likely that KGF plays an important role in epithelial-mesenchymal interactions to support epithelial repair, therefore it would be of interest to investigate the expression of the KGFR in this model of 16HBE 14o$^-$ cells.

The TGF-β1 ELISA was used to measure active and total TGF-β1 in cell culture supernatants. The concentration of latent TGF-β1 was calculated from the active and total concentrations. Mechanical wounding did not elicit any changes in the concentration of active TGF-β1 in cell culture supernatants. However, following acid-activation of samples, the concentration of total TGF-β1 was significantly increased at 4 hours *post*-wounding, whereby 30.3 pg/ml TGF-β1 was detected in cell culture supernatants. Since the active form of TGF-β1 remained constant, the increase in total TGF-β1 refers to the latent form of this growth factor. In a time-lapse model of wound repair of a 16HBE 14o$^-$ monolayer, stimulation with 0.25 ng/ml TGF-β1 resulted in a significantly enhanced migratory rate over 4 hours, whereas TGF-β2 was not found to play a role in cell migration during repair of 16HBE 14o$^-$ cells (Howat *et al.*, 2002). Thus, the concentration of TGF-β1 detected in the current study is considerably lower

than that required to promote wound repair. In the same study, Howat *et al.* demonstrated an increase in active TGF-β1 2 hours *post*-wounding, however, this accumulation was only seen in cultures in which serum containing medium was used, indicating that latent TGF-β1 in the serum was the substrate for activation. TGF-β is produced by bronchial epithelial cells, mesenchymal cells, eosinophils and macrophages and has regulatory effects on extracellular matrix production in the lung and hence epithelial repair (Coker *et al.*, 1997). The use of serum-containing media in cell culture models of wound repair reflects the *in vivo* situation where immunocytochemistry has shown that the majority of TGF-β1 is found in the subepithelial compartment (Redington *et al.*, 1998). Therefore, it is likely that the presence of serum in Howat's model enhanced the ability of the 16HBE 14o$^-$ cells to convert latent TGF-β1 into its active form in response to wounding. In the absence of serum, 16HBE 14o$^-$ cells have the capacity to produce latent TGF-β1 in response to wounding. Hence, TGF-β1 is unlikely to contribute to wound repair in this model.

In hindsight, the design of the cell experiments to generate supernatants for both the growth factor and IL-8 assays was flawed as they displayed a lack of time-matched controls. If these experiments were to be repeated, they would include both unwounded and wounded cell cultures for each time-point investigated.

HGF is demonstrated to be a potent biological mediator of mitogenesis, motogenesis morphogenesis and differentiation in a variety of cell types, including bronchial epithelial cells (Jiang *et al.*, 1997). The addition of 20 ng/ml HGF has been reported to enhance repair of nasal epithelial cells (Zahm *et al.*, 2000). HGF was not detected in cell culture supernatants by ELISA (data not shown) at baseline or in response to wounding. Furthermore, immunohistochemistry demonstrated that HGF is not expressed by 16HBE 14o$^-$ cells of intact or wounded monolayers. However, the same cell type demonstrated strong expression of the HGF (*c-Met*) receptor, confirming reports in the literature that the *c-Met* receptor is predominantly expressed on the surface of various epithelial cell types including those of the lung (Shen *et al.*, 1997). HGF expression is predominantly confined to the mesenchyme. In human bronchial epithelial cells, HGF appears to act mainly as a paracrine factor produced by mesenchymal cells such fibroblasts, which interact with epithelial cells (Singh-Kaw *et al.*, 1995; Sonnenberg *et al.*, 1993). However, there is a conflicting report that demonstrates that normal human

bronchial epithelial cells can produce HGF in culture as an autocrine motogenic factor (Tsao *et al.*, 1993). The role of HGF in immune-mediated disorders such as asthma is unclear, however, there is a report to suggest that HGF might be effective in preventing asthma exacerbation preceded by viral infection as well as suppressing antigen-induced Th2-type immune response in an *in vivo* mouse model (Okunishi *et al.*, 2005). *In vivo*, it is likely that HGF mediates its effects by epithelial-mesenchymal interactions. A recent study using a primary HBE-fibroblast co-culture model has demonstrated that HGF secreted by subepithelial fibroblasts modulates HBE cell differentiation (Myerburg *et al.*, 2007). Thus, it would be of interest to develop a co-culture with HBE cells and fibroblasts to investigate the effect of endogenous HGF on wound repair.

Interestingly, 16HBE 14o$^-$ cells demonstrated positive, specific immunostaining for FXIII, a highly novel observation. Stimulation with 100 ng/ml HGF resulted in the diffusion of FXIII from perinuclear sites to the plasma membrane of the cells. Since FXIII plays an important role in the final step of fibrin formation by cross linking the fibrin clot, it appears that HGF may be involved in the modulation of this process, by stimulating the diffusion of this factor to the periphery of the cell and thereby making it more available to perform its duty.

Savla *et al.* demonstrated that exogenously added PGE_2 (10 μg/ml) stimulated wound repair in an *in vitro* model of 16HBE 14o$^-$ cells and that the COX inhibitor indomethacin retarded repair (Savla *et al.*, 2001). Since PGE_2 was reported to enhance wound repair, its release from 16HBE 14o$^-$ cells and effects on fibrin formation were investigated. Analysis of cell culture supernatants by ELISA indicated that endogenous PGE_2 was present at a low concentration of 9.4 pg/ml and that its release was unaffected by wounding at 20 minutes or 2 hours. Immunoblot analysis indicated that exogenous PGE_2 in the concentration range of 0-20 μg/ml did not elicit any changes in the concentrations of fibrinogen, FXIII or D-dimers in cell culture supernatants of the intact or wounded monolayers at 20 minutes or 2 hours in this model, suggesting that PGE_2 is not involved in the formation of fibrin to support bronchial epithelial repair. According to Savla *et al.*, the effects of PGE_2 are mediated *via* EP-1 and EP-4 receptors; however, mRNA for these receptors was not detected in 16HBE 14o$^-$ cells (*Chapter 6*) under the current culture conditions.

IL-8 is is a neutrophil chemotactic factor that is produced in abundance by mononuclear phagocytic cells, as well as a number of non-inflammatory cells including fibroblasts. bronchial and alveolar epithelial cells. Synthesis of IL-8 by fibroblasts and alveolar epithelial cells involves stimulus specificity, as the production of this mediator by non-inflammatory cells is dependent upon an initial host response. Alveolar macrophages appear to play a central role in inflammatory airway diseases by generating factors, such as IL-1 and TNF, which are potent stimuli for the induction of IL-8 by lung fibroblasts and alveolar epithelial cells. This cascade-like interaction may lead to the rapid production of significant quantities of IL-8 by the lung and subsequent recruitment of neutrophils to the airway lumen; and may be particularly important in asthma which is associated with abundant expression of this inflammatory chemokine (Kunkel *et al.*, 1991).

In addition to their role in fibrin formation, there is broad evidence to suggest that coagulation factors VII, Xa and thrombin may evoke inflammatory responses by stimulating expression of inflammatory mediators, such as IL-8 (*figure 3.36*) (Daubie *et al.*, 2006; Schulman, 2003). When coagulation is initiated, cell signalling occurs simultaneously. TF-induced signalling can occur *via* the cytoplasmic tail of TF or the proteolytic activity of FVII in association with TF (Pendurthi *et al.*, 2002). TF-FVII proteolytic activity mediates signalling *via* PAR-2 receptor, to induce the expression of a number of inflammatory mediators, including IL-8. There is general agreement that thrombin activates PAR-1 and PAR-4 to stimulate the release of IL-8, however, it is unclear whether FXa stimulates the release of IL-8 by effector cell protease receptor 1 (EPR-1) (Cirino *et al.*, 1997), PAR-1 (Schulman, 2003) or PAR-2 (Camerer *et al.*, 2000). Since this study demonstrates activation of the coagulation cascade, it was of interest to measure the levels of IL-8 in cell culture supernatants of 16HBE 14o$^-$ in response to wounding in order to investigate a link between coagulation and inflammation. The concentration of IL-8, as measured by ELISA, demonstrated a significant increase in IL-8 at 4 hours *post*-wounding compared to the unwounded control. This is indicative of an effect on IL-8 expression rather than release of the pre-formed mediator. Interestingly, FVII mRNA expression was increased 4 hours *post*-wounding as demonstrated in *Chapter 6*, which might indicate that IL-8 expression was dependent on TF:FVII signalling *via* PAR-2.

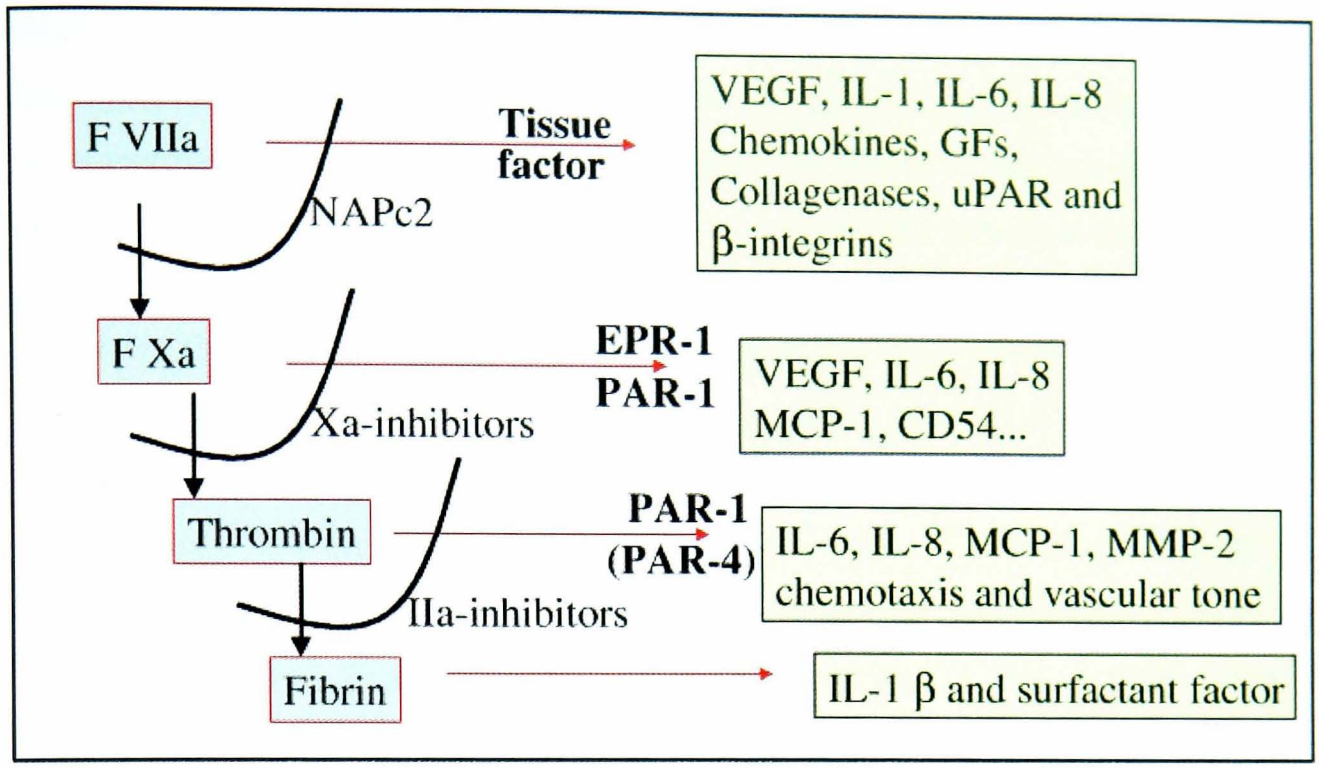

Figure 3.36. The coagulation cascade as a vertical flow chart and horizontal reactions outside the coagulation system indicating a role for these factors in inflammation. VEGF, vascular endothelial growth factor; IL, interleukin; GF, growth factor; uPAR, urokinase type plasminogen activator receptor; MCP, monocyte chemotactic protein; CD54, ligand for a cell adhesion receptor; MMP, matrix metalloproteinase (Schulman, 2003).

IL-8 stimulates the recruitment of neutrophils in the airways. Severe asthma is typically associated with an abundance of IL-8 and neutrophils and subsequent tissue destruction and airway remodelling (Heinzmann *et al.*, 2004). In severe asthma and exacerbations of asthma, neutrophil elastase is considered to play a role in the chronic inflammation associated with the uncontrolled remodelling response (Holgate *et al.*, 2006). Elastase has a number of effects in the airway, as discussed in *section 3.1.11*. In the normal airway, elastase may remove fibrin (Kolev *et al.*, 1996) and prevent fibrosis; and cleave a number of coagulation factors, including FVII (Anderssen *et al.*, 1993), FX (Turkington, 1991) and FXIII (Klingemann *et al.*, 1982) following wound repair. Since these coagulation factors have been detected in this model, it was of interest to investigate the effects of elastase on the levels of these factors in cell culture supernatants. Elastase (0.5 U/ml) significantly reduced the levels of fibrinogen and FXIII in the wounded monolayers at 20 minutes. Elastase is known to inactivate PAR-2 (Dulon *et al.*, 2003); however, PAR-2 is demonstrated to strongly enhance the release of coagulation factors in the subsequent repair response. Therefore, this effect may signify either elastase-mediated inhibition of PAR-stimulated release of these factors or damage to the factors induced by proteolytic cleavage. However, 500 nM elastase was used to

disarm PAR-2, therefore low concentrations of neutrophil elastase such as those employed in the current study are more likely to degrade coagulation factors. Elastase was subsequently shown to inhibit wound repair in *Chapter 4*, providing evidence that the stimulated release of coagulation factors are important in bronchial epithelial repair.

Studies by Erjefalt *et al.*, demonstrated that immediately after removal of the guinea pig epithelium *in vivo*, there was evidence of extravasation and bulk entry of plasma from the microcirculation beneath the denuded, but intact basement membrane, resulting in the formation of a fibrin gel matrix to facilitate epithelial restitution. Erjefalt *et al.* concluded that the coagulation factors involved in the formation of fibrin were exclusively derived from the bronchial microcirculation. In the current serum-free model, extrinsic coagulation factors that mediate fibrinogenesis were detected at baseline and released from 16HBE $14o^-$ cells in response to wounding. Moreover, the significant release of D-dimers at 2 hours *post*-wounding signifies that cross-linked fibrinogen or fibrin was formed in response to epithelial damage. The early formation of a fibrin(ogen) matrix supports Erjefalt's findings *in vivo.* However, in this reductive model that investigates the role of bronchial epithelial cells *alone*, there is novel evidence that these cells are themselves a source of coagulation factors that are released in response to wounding, independently of plasma-derived proteins or any other influences that may be present *in vivo*. Erjefalt *et al.*, also demonstrated the early accumulation of neutrophils in areas of epithelial damage induced mechanically or by allergen challenge. This correlates with the significant increase in IL-8 release 4 hours *post*-wounding in this model since IL-8 is a major chemoattractant for neutrophils. Furthermore, D-dimers can be proinflammatory and stimulate the recruitment of neutrophils (Wojtecka-Lukasik *et al.*, 1992). Dysregulation of D-dimer production by the bronchial epitheium could lead to amplification of the inflammatory process, therefore, it would be of interest to investigate the levels of D-dimers at later time-points, or indeed to measure the activity of plasmin, the fibrinolytic enzyme, over a range of time-points.

Chapter 4.

Role of Coagulation Factors in Wound Repair

4. The role of coagulation factors in wound repair

4.1. Introduction

In vivo studies in the guinea pig lung have demonstrated that bronchial epithelial repair following injury is both rapid and efficient and is dependent on the formation of a provisional fibrin matrix which is believed to be exclusively plasma-derived (Erjefalt *et al.*, 1994). However, data presented in *Chapter 3* has demonstrated the ability of bronchial cells to release coagulation cascade factors in response to injury, *independently* of plasma proteins. Furthermore, the increase in the release of D-dimers from 16HBE 14o$^-$ cells *post*-wounding indicates that wounding initiates fibrin formation, since D-dimers are a marker of both fibrinolysis and fibrinogenesis.

Fibrinogen has long been established as serving an important haemostatic role *in vivo* because it is converted from a soluble plasma protein to an insoluble fibrin gel by the action of thrombin and FXIIIa (Doolittle, 1981). The fibrin clot provides a scaffold for cell migration. Fibrinogen is predominantly synthesised by hepatocytes and its expression is upregulated in response to inflammation as part of the systemic acute phase response (Lawrence *et al.*, 2004). However, cultured lung epithelial (A549) cells have been demonstrated to synthesise and secrete fibrinogen following stimulation with IL-6 and dexamethasone (Haidaris, 1997). It was reported that alveolar epithelial cell-derived fibrinogen assembled into an ECM at sites of epithelial damage and the resultant matrix fibrinogen was demonstrated to significantly enhance wound repair, independently of exogenously added growth factors.

Fibrinogen and fibrin can bind collagen IV (Jones *et al.*, 1988), the major component of the basement membrane (Legrand *et al.*, 1999) and it is likely that this provides a mechanism of attachment of the provisional matrix to the basement membrane. Integrins are required for the anchorage of cells and during epithelial wound repair there is an up-regulation of the integrins that bind to fibrin(ogen), fibronectin and vitronectin (Pilewski *et al.*, 1997). Fibronectin is an important constituent of the provisional matrix and it promotes the chemotaxis of epithelial cells and fibroblasts for repair (Polito *et al.*, 1998; Yamada, 2000).

The lack of a fibrin provisional matrix compromises wound repair and an unstable provisional matrix due to FXIII deficiency has been demonstrated in a number of diseases, such as venous leg ulcers (Herouy *et al.*, 2000), Crohn's disease (Chamouard *et al.*, 1998) and ulcerative colitis (D'Argenio *et al.*, 2000), resulting in defective epithelial repair. In addition to fibrin and integrins, a number of factors including TFF peptides, NO, relaxin and glycoproteins are established to facilitate cell migration and wound repair. Moreover, following epithelial injury, several members of the MMP family are induced and activated. In particular, MMP-9 is typically produced by the bronchial epithelium and activated during injury (Bove *et al.*, 2007). Migrating bronchial epithelial cells *in vivo* express increased levels of MMP-9 (Buisson *et al.*, 1996) located at the edge of lamellaepodia where it acts to modify collagen IV (Legrand *et al.*, 1999). NO has been demonstrated to promote epithelial cell migration and wound repair *via* increased expression and activation of MMP-9 (Bove *et al.*, 2007). Similarly, expression of MMP-7, also known as matrilysin is upregulated in migrating epithelial cells and its activity has been associated with epithelial wound repair (Parks *et al.*, 2001). Moreover, TGF-β1 has been shown to enhance epithelial wound repair *via* upregulation of MMP-2 (Lechapt-Zalcman *et al.*, 2006). However, little is known about the contribution of locally produced coagulation factors.

A number of the proteases within the coagulation cascade have been shown to specifically act upon PARs. The coagulation response is therefore not just for haemostatic control and wound repair, but it also influences epithelial cell signalling. There are at least four coagulation cascade proteases involved in the coagulation pathway that have the capacity to activate PAR receptors, namely FVIIa, FXa, thrombin and activated protein C (Pendurthi *et al.*, 2002), in addition to the ternary TF:FVIIa:FXa complex (Chambers *et al.*, 2002). To date, four PARs (PAR-1, PAR-2, PAR-3 & PAR-4) have been isolated and the table below shows a comparison of the serine proteases that activate the receptors (Chambers *et al.*, 2002; Macfarlane *et al.*, 2001). Refer to *Chapter 5* for a full description of these receptors.

Proteinase	Source	PAR
Thrombin	Blood	PAR-1, PAR-3 and PAR-4
Trypsin	Epithelium	PAR-1, PAR-2 and PAR-4
Mast cell tryptase	Mast cells, basophils	PAR-2
TF-FVIIa-FXa	Blood	PAR-1 and PAR-2
FVIIa	Blood	PAR-2
FXa	Blood	PAR-1 and PAR-2
Neutrophil proteinase-3	Neutrophils	PAR-2
HAT	Epithelium	PAR-2
Der P1, Der P3, Der P9	Dust mite allergen	PAR-2
Cathepsin G	Monocytes, neutrophils	PAR-4
Matriptase	Epithelium	PAR-2
MMP-1	Epithelium	PAR-1

Table 4.1. A Summary of PAR activating proteinases in the lung.

Endogenous activators of PAR-1 include thrombin, FXa, TF-FVIIa-FXa and activated protein C. PAR-2 may be activated by trypsin, coagulation factors VIIa and Xa and TF-FVIIa-FXa. *In vivo* models have shown that trypsin released from the epithelium can initiate powerful bronchoprotection in the airways *via* activation of PAR-2 and release of the cyclooxygenase product PGE_2. PGE_2 is presumed to have a bronchoprotective effect by stimulating smooth muscle relaxation, and inhibiting stimulated bronchoconstriction. The transient ternary TF-FVIIa-FXa complex also activates PAR-1 and PAR-2 supporting the notion that the TF-FVIIa-initiated coagulation cascade is inseparably linked to PAR activation and cell signalling as reviewed by Chambers and Laurent (2002).

Pseudomonas aeruginosa, a major lung pathogen in cystic fibrosis (CF), is known to secrete elastolytic metalloproteinase (EPa), also known as elastase. EPa was demonstrated to disarm PAR-2, thereby preventing its subsequent activation by trypsin. Inactivation of PAR-2 occurred through proteolysis of the extracellular N-terminal domain downstream from the trypsin cleavage/activation site (Dulon *et al.*, 2005). Since one of the roles of PAR-2 in the lung is to contribute to the control of the innate response of this organ to invading pathogens like *Pseudomonas aeruginosa*, and to the preservation of respiratory functions through production of mediators such as IL-8 and PGE_2, its inactivation by EPa in an infectious/inflammatory environment as demonstrated in CF, could provide an explanation of some of the pathogenic activities associated with this microorganism.

The majority of the literature to date describes the role of FXa on the endothelium. However, FXa activity has been demonstrated on the surface of alveolar A549 cells that could be inhibited with the use of the synthetic FXa inhibitor DX9065a (Nakata *et al.*, 1998). An immunohistochemical study demonstrated that FXa expression is also localised to the bronchial epithelium (Yamada *et al.*, 1996). EPR-1 is a cell surface receptor for FXa (Nicholson *et al.*, 1996) that is involved in localising FXa to the endothelial cell surface leading to the formation of the prothrombinase complex (Bouchard *et al.*, 1997; Chambers *et al.*, 2002). However, its expression on bronchial epithelial cells has not yet been reported. Binding of FXa to EPR-1 has been demonstrated to stimulate the release of PDGF and enhance the mitogenic effect of FXa on lung fibroblasts (Blanc-Brude *et al.*, 2005) and smooth muscle cells (Herbert *et al.*, 1998). Moreover, EPR-1 has previously been reported to be necessary to localise FXa in close proximity to the endothelial cellular membrane in order to selectively cleave and activate PAR-2 (Bono *et al.*, 2000).

FXa is an activator of both PAR-1 and PAR-2, which are expressed in the normal bronchial epithelium (Lan *et al.*, 2002; Riewald *et al.*, 2001). Several studies have demonstrated that FXa stimulates fibroblast proliferation and induces collagen production through activation of PAR-1 (Blanc-Brude *et al.*, 2005; Chambers *et al.*, 2002). A role for PAR-1 in tissue fibrosis has been identified (Chambers *et al.*, 2002) and activation of this receptor by FXa might potentially contribute to airway remodelling by regulation of fibroblast proliferation and collagen deposition. Thus, FXa can regulate asthmatic events. A recent study using a murine model has demonstrated that FXa is involved in airway remodelling (Shinagawa *et al.*, 2007). The presence of FXa led to increased mucin production through upregulation of amphiregulin in lung epithelial cells. It was reported that FXa might also contribute to remodelling by activation of PARs and a resultant increase in fibroblast proliferation and collagen production.

Whether the activation of PARs results in inflammation and/or wound repair is presently unclear. Moreover, the relationship between PAR activation, coagulation factor release and fibrin formation is not known. It was therefore of interest to investigate a functional role for the coagulation factors and fibrin formation in wound

repair of bronchial epithelial cells using a model of 16HBE 14o$^-$ and NHBE cells, which examines the role of bronchial epithelial cell-derived coagulation factors in the repair response.

4.2. Aims and objectives

Having established that TF, fibrinogen and FXIII were expressed by 16HBE 14o$^-$ cells at the protein level, it was of interest to investigate the roles of these coagulation cascade proteins in wound repair of both 16HBE 14o$^-$ and NHBE cells. This was to be achieved by the use of corresponding neutralising antibodies to these proteins. Further aims were to investigate the roles of FXa and thrombin in wound repair of 16HBE 14o$^-$ cells by the use of selective inhibitors. Results in *chapter 3* demonstrated that the concentration of PGE_2 in supernatants derived from 16HBE 14o$^-$ cells was negligible, however, PGE_2 has previously been reported to enhance repair of 16HBE 14o$^-$ cells, therefore, it was of interest to confirm whether or not PGE_2 was involved in wound repair of 16HBE 14o$^-$ cells in the current model. This was to be established by the use of the COX inhibitor indomethacin. Finally, since neutrophil elastase reduced the supernatant levels of coagulation factors in 16HBE 14o$^-$ cells, it was imperative to determine the effects of this inflammatory mediator on wound repair.

4.3. Methods

4.3.1. Culture of 16HBE 14o$^-$ cells

16HBE 14o$^-$ cells were cultured for 48 hours in 24-well plates as described in *section 2.2.1* until fully confluent. At confluence, cells were washed by adding 500 µl of 1X PBS (without calcium and magnesium) to each well. To quiesce the cells, 500 µl of serum-free MEM-ITS (basal MEM containing 2 mM L-glutamine and 100 U/ml antibiotic/antimycotic, supplemented with 1% ITS) was added to each well and the plates were incubated for 16 hours at 37°C. Prior to each experiment, cells were washed by adding 500 µl per well of 1X PBS, and 250 µl of fresh, serum-free MEM-ITS was added to each well.

4.3.2. Culture of NHBE cells

NHBE cells were cultured for 5 days in 10 μg/cm^2 collagen IV-coated 24-well plates, as described in *section 2.2.3* until fully confluent. At confluence, cells were washed by adding 500 μl of 1X PBS (without calcium and magnesium) to each well. To quiesce the cells, 500 μl of serum-free BEGM-ITS (basal MEM containing 2 mM L-glutamine and 100 U/ml antibiotic/antimycotic, supplemented with 1% ITS) was added to each well and the plates were incubated for 16 hours at 37°C. Prior to each experiment, cells were washed by adding 500 μl per well of 1X PBS, and 250 μl of fresh, serum-free BEGM-ITS was added to each well.

4.3.3. Effect of neutralising coagulation factor antibodies on repair of epithelial monolayers

A concentration range of: 0, 1, 5 and 10 μg/ml (for 16HBE 14o$^-$ cells) and 0, 0.1, 0.5 and 1 μg/ml (for NHBE cells) of: a mouse monoclonal antibody to human TF (1 mg/ml stock dissolved in sterile deionised water; American Diagnostica Inc; supplied by Axis-Shield, Dundee, UK); rabbit polyclonal antibody to human fibrinogen (IgG-peroxidase conjugate, 1 mg/ml stock dissolved in 1X PBS: Affinity Biologicals Inc; supplied by Quadratech, Surrey, UK) or sheep antibody to human FXIII (subunit A, IgG-peroxidase conjugate, 2 mg/ml stock dissolved in DMSO: Affinity Biologicals Inc; supplied by Quadratech, Surrey, UK) and corresponding mouse IgG (purified mouse IgG, 1 mg/ml stock dissolved in 1X PBS: Sigma, Dorset, UK), rabbit IgG (purified rabbit IgG, 1 mg/ml stock dissolved in 1X PBS: Sigma, Dorset, UK) or sheep IgG (purified sheep IgG, 1 mg/ml stock dissolved in 1X PBS: Sigma, Dorset, UK) was added to cells in a volume of 250 μl per well and the plate was incubated for 30 minutes at 37°C. Wound repair was then carried out as described in *section 4.3.10*.

4.3.4. Stimulation of 16HBE 14o$^-$ cells with exogenous FXa

A concentration range of: 0, 50, 100 and 200 nM FXa (20 mM stock dissolved in 1X PBS: Calbiochem®, supplied by Merk Chemicals LTD, Nottingham, UK) was added to

cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. Wound repair was then carried out as described in *section 4.3.10.*

4.3.5. Effect of thrombin and FXa inhibitors on repair of 16HBE 14o$^-$ monolayers

A concentration range of: 0, 0.05, 0.1, 0.2, 0.5 and 1 µM of a selective thrombin inhibitor (UK-156,406; 10 mM stock dissolved in DMSO: Pfizer Global R&D compound), selective FXa inhibitor (UK-220,047-01, the Daiichi compound DX9065a: 10 mM stock dissolved in DMSO; supplied by Pfizer Global R&D) or alternative more selective FXa inhibitor (PD-031, 10 mM stock dissolved in DMSO: Pfizer Global R&D compound) was added to cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. DMSO controls at a final concentration of 0.0005, 0.001, 0.002, 0.005 and 0.01 % corresponding to 0.05, 0.1, 0.2, 0.5 and 1 µM inhibitor respectively were also included. Wound repair was then carried out as described in *section 4.3.10.*

4.3.6. Effect of indomethacin on repair of 16HBE 14o$^-$ monolayers

A concentration range of 0, 0.01, 0.1, 1, 3 and 10 µM indomethacin (1 mM stock dissolved in 1% DMSO: Sigma, Dorset, UK) was added to cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. A concentration range of up to 0.01% DMSO, corresponding to 10 µM indomethacin was also included. Wound repair was then carried out as described in *section 4.3.10.*

4.3.7. Effect of PGE_2 on repair of 16HBE 14o$^-$ monolayers

A concentration range of: 0, 0.1, 1, 5, 10 and 20 µg/ml PGE_2 (2 mg/ml stock dissolved in 50% ethanol/50% MEM-ITS: Sigma, Dorset, UK) was added to cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. Ethanol controls at a final concentration of 0.0025, 0.025, 0.125, 0.25 and 0.5 % corresponding to 0.1, 1, 5, 10 and 20 µg/ml PGE_2 respectively were also included. Wound repair was then carried out as described in *section 4.3.10.*

4.3.8. Effect of neutrophil elastase on repair of 16HBE 14o$^-$ monolayers

A concentration range of: 0, 0.1, 0.03, 0.1, 0.3 and 0.5 U/ml neutrophil elastase (3 U/ml stock dissolved in 1X PBS: Sigma, Dorset, UK) was added to cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. Wound repair was then carried out as described in *section 4.3.10.*

4.3.9. Effect of mitomycin C on repair of 16HBE 14o$^-$ monolayers

A final concentration of: 1 µg/ml mitomycin C (1mg/ml stock dissolved in deionised H_2O: Sigma, Dorset, UK) was added to cells in a volume of 250 µl per well and the plate was incubated for 30 minutes at 37°C. Wound repair was then carried out as described in *section 4.3.10.*

4.3.10. Wound repair of 16HBE 14o$^-$ and NHBE cells

Cells were mechanically wounded with a P2 Gilson pipette tip, one horizontal scrape per well. Immediately after wounding, one photograph per well was taken of a representative section of wound using the inverted microscope (Leica Microsystems, Wetzlar, Germany) and Ricoh XR-3000 35 mm camera. Cells were returned to the incubator, at 37°C for 13 hours. A second set of photographs was then taken of the same section of wound. Photographs were developed and wound repair was analysed by taking 10 measurements across the wound and the % original wound width was calculated at 13 hours. Supernatants and cells were harvested and stored at -80°C, as described in *section 2.2.4.*

4.3.11. Immunoblotting for fibrinogen and FXIIIA

Following wound repair in 16HBE 14o$^-$ and NHBE cell layers, supernatants were removed at 13 hours and 20 hours respectively and centrifuged for 7 minutes at 670 x *g* (ALC PK120 Centrifuge, Winchester Virginia, USA) to remove cell debris. Cell culture

supernatants were then analysed by immunoblot for fibrinogen and FXIIIA as described in *section 3.3.3*.

4.4. Results

4.4.1. Effect of mitomycin C

In order to determine whether wound repair in this model was occurring by cell spreading and migration or proliferation to repopulate the denuded area, the effect of mitomycin C (MMC), a proliferation inhibitor was investigated. MMC is a chemotherapeutic agent that prevents DNA double-strand separation during cell replication by forming covalent cross-links between DNA opposite strands, allowing RNA and protein synthesis to continue and arresting cells in G1, S and G2 phases of the cell cycle (Barlogie *et al.*, 1980; Tomasz *et al.*, 1987). A final concentration of 1 μg/ml was chosen as a previous *in vitro* study demonstrated that treatment of retinal pigment epithelial cells with 1 μg/ml MMC was sufficient to arrest cell proliferation without inducing apoptosis, which occurred at the higher concentration of 10 μg/ml MMC (Kang *et al.*, 2001).

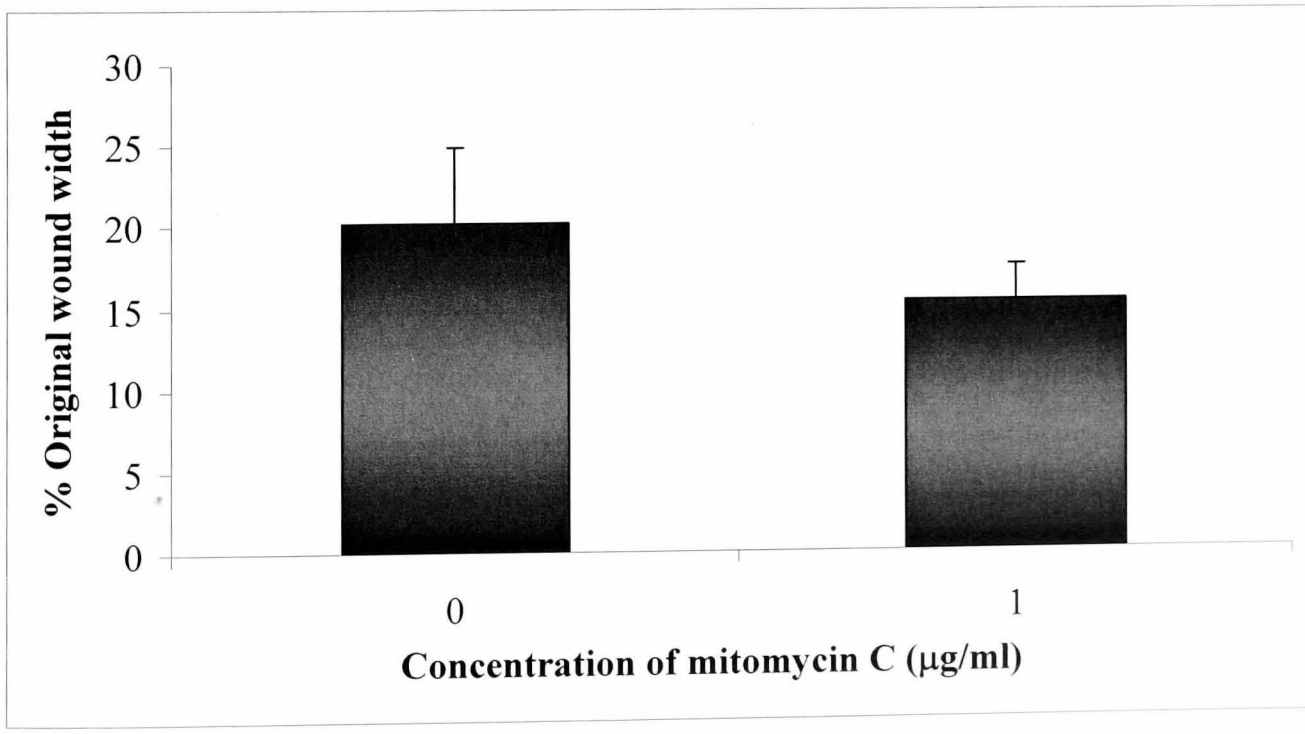

Figure 4.1. Effect of MMC on wound repair of 16HBE 14o$^-$ cells at 9 hours. Data represent mean ± SEM (*n=6*).

The % original wound width in the untreated 16HBE 14o$^-$ cell cultures at 9 hours was 20.19 ± 4.68%. Treatment of cells with 1 μg/ml MMC reduced the wound width to 15.4 ± 2.18%. There was no significant effect of MMC on wound repair.

4.4.2. Role of TF in 16HBE 14o$^-$ cells

TF initiates the coagulation cascade and was shown to be constitutively expressed and active in 16HBE 14o$^-$ cells (*section 3.4.1.1*), therefore the effect of a function blocking antibody to human TF in this model of 16HBE 14o$^-$ wound repair was investigated.

4.4.2.1. Wound repair

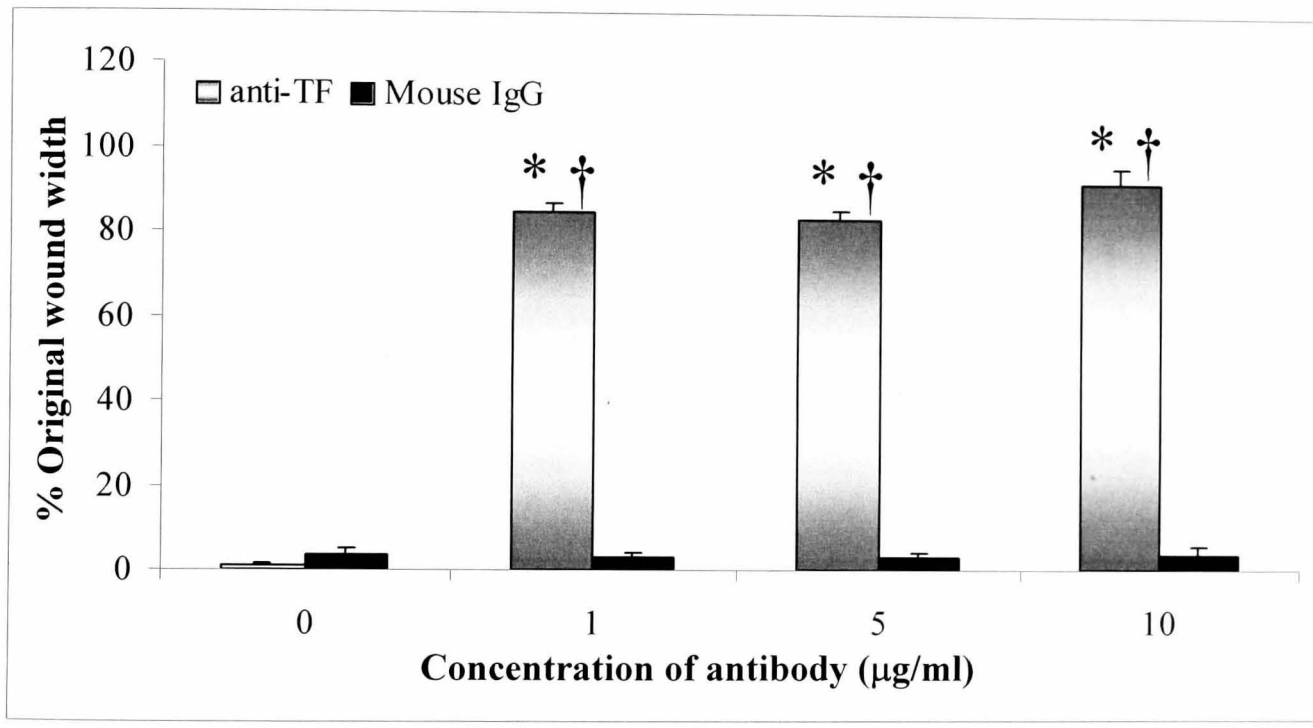

Figure 4.2. Effect of anti-TF on repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.0001, compared to no-antibody control and † indicates P<0.0001 compared to non-immune mouse immunoglobulin.

In the absence of anti-TF, the % original wound width, i.e. the % of the wound still open after 13 hours was 1.15 ± 0.57 %. Thus, the wound had almost completely repaired in 13 hours. With the addition of 1 μg/ml anti-TF, wound repair was significantly inhibited (84.60 ± 2.40 %) compared to both no antibody control and non-immune mouse immunoglobulin (3.29 ± 0.89 %), with no further significant effect at higher antibody concentrations. There was no effect of the non-immune mouse immunoglobulin on wound repair.

4.4.2.2. Concentration of fibrinogen in cell culture supernatants

Because TF initiates the coagulation cascade, it was of interest to investigate the levels of other coagulation factors of the extrinsic cascade, including fibrinogen and FXIIIA in cell culture supernatants at 13 hours, following inhibition of wound repair by anti-TF.

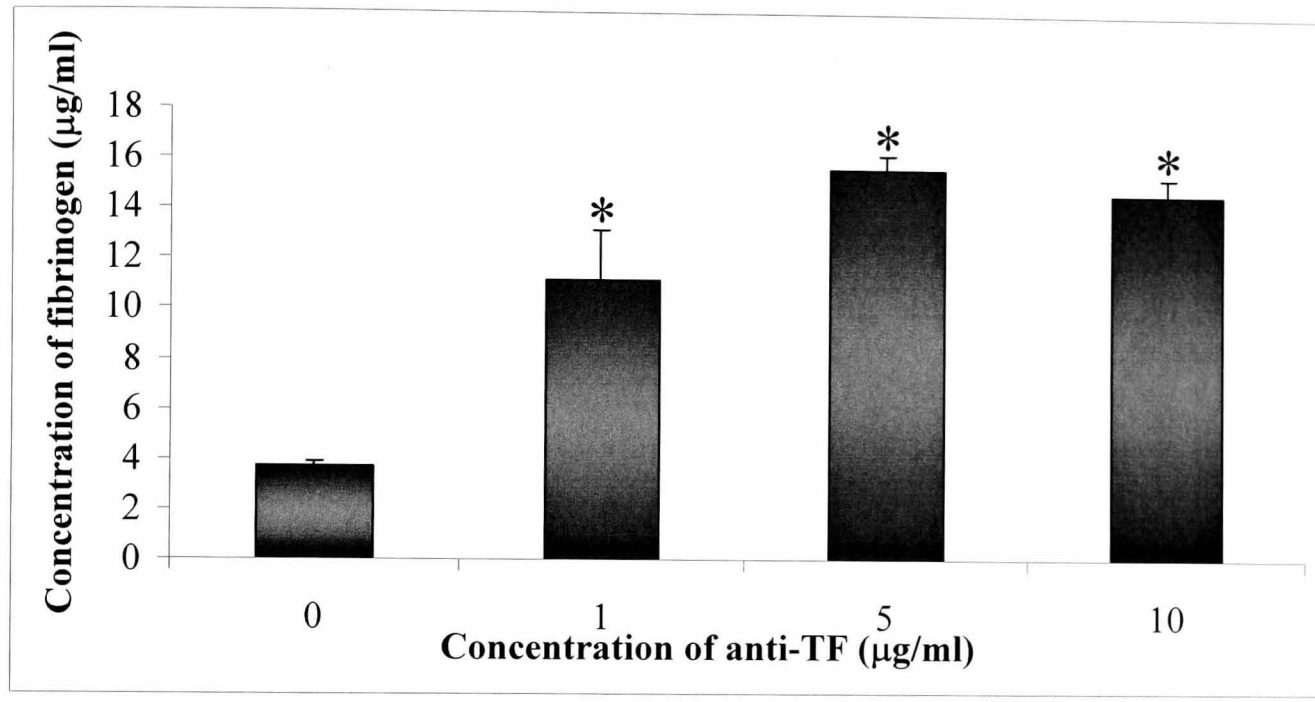

Figure 4.3. Effect of anti-TF on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells 13 hours *post*-wounding. Data represent mean ± SEM (*n=3*). * Indicates P<0.05 compared to no antibody control.

Inhibition of wound repair by anti-TF led to the accumulation in culture supernatants of fibrinogen released from wounded cell monolayers. The concentration of fibrinogen in culture supernatants was significantly increased in the presence of 1 µg/ml anti-TF (11.22 ± 1.96 µg/ml) compared to the no antibody control (3.68 ± 0.19 µg/ml). However, there was no further significant increase in the presence of 5 µg/ml anti-TF (15.60 ± 0.63 µg/ml) or 10 µg/ml anti-TF (14.66 ± 0.68 µg/ml).

4.4.2.3. Concentration of FXIIIA in cell culture supernatants

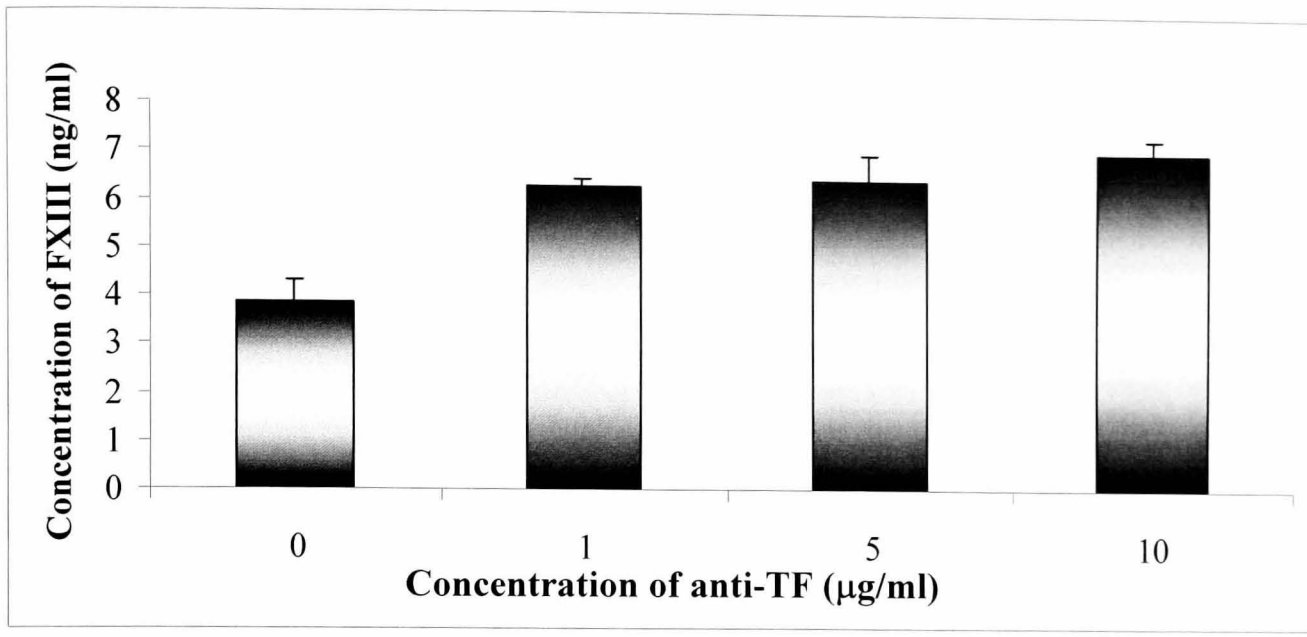

Figure 4.4. Effect of anti-TF on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells 13 hours *post*-wounding. Data represent mean ± SEM (*n=3*).

Analysis of cell culture supernatants following inhibition of wound repair by anti-TF indicated a trend of increasing concentration of FXIIIA with increasing concentration of anti-TF. In the presence of 10 μg/ml anti-TF, the concentration of FXIIIA (7.02 ± 0.27 ng/ml) was almost double that of the no-antibody control (3.83 ± 0.48 ng/ml), however, the effect was not significant (p=0.107).

4.4.3. Role of fibrinogen in 16HBE 14o$^-$ cells

TF not only activates the extrinsic coagulation cascade, but also generates proteases that activate PARs that are present on bronchial epithelial cells. A direct role for cross-linked fibrin formation in the wound repair process was therefore investigated using antibodies to fibrinogen (*section 4.4.3.1*) and FXIIIA (*section 4.4.4.1*).

4.4.3.1 Wound repair

Fibrinogen is the precursor for fibrin, which has a role in bronchial epithelial repair. The concentration of fibrinogen in cell culture supernatants was shown to increase, dependent on the extent of wounding (*section 3.4.2.1*) and accumulated when wound

repair was inhibited (*section 4.4.2.2*). Therefore, the effect of an antibody to fibrinogen was investigated in this model of 16HBE 14o$^-$ wound repair.

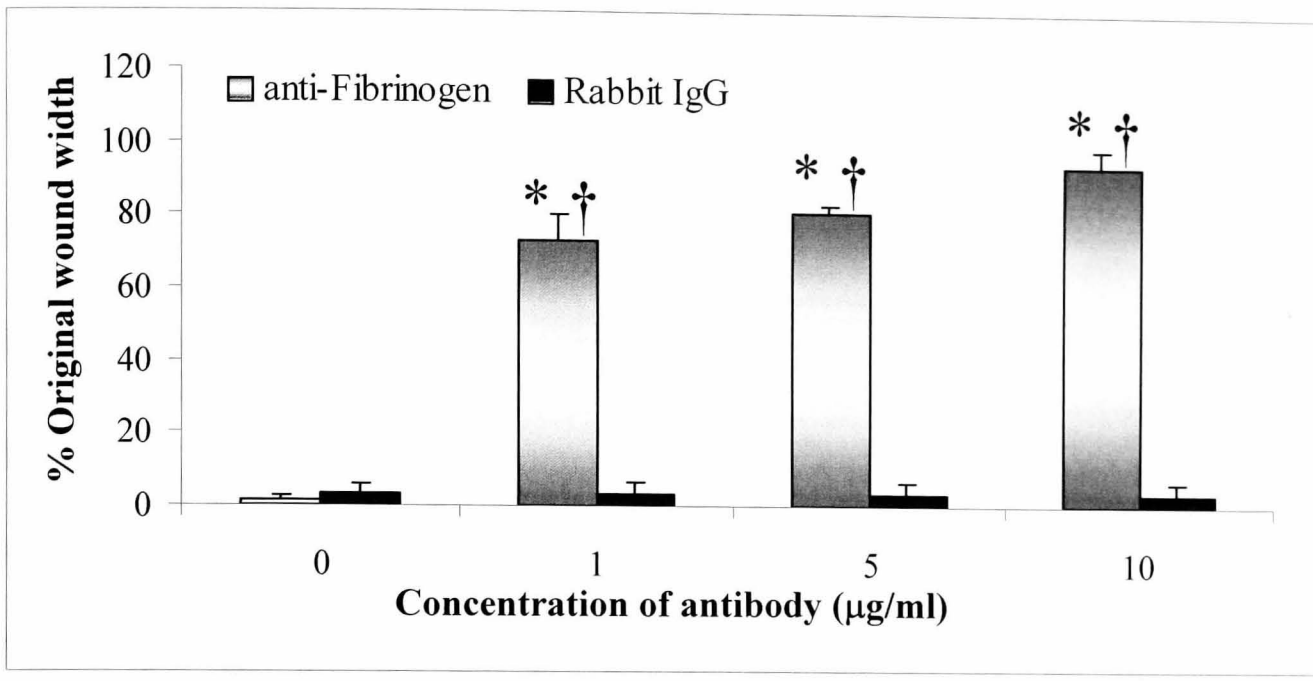

Figure 4.5. Effect of anti-fibrinogen on repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.0001, compared to no-antibody control and † indicates P<0.0001 compared to non-immune rabbit immunoglobulin.

The % original wound width in no antibody control wells was 1.55 ± 0.78 %, indicating almost complete repair at 13 hours. Bronchial epithelial repair was significantly inhibited with the addition of 1 μg/ml anti-fibrinogen (72.72 ± 0.76 %) compared to both no antibody control and non-immune rabbit immunoglobulin (3.37 ± 2.75%). Wound repair was further reduced in the presence of 5 μg/ml anti-fibrinogen (81.07 ± 1.91%) and 10 μg/ml anti-fibrinogen (94.03 ± 4.44%); however, there was no further significant effect at these concentrations. Non-immune rabbit immunoglobulin had no effect on bronchial epithelial repair.

4.4.3.2. Concentration of fibrinogen in cell culture supernatants

Since fibrinogen and FXIIIA are involved in the final step of fibrin formation, it was of interest to investigate the levels of these factors in cell culture supernatants 13 hours following inhibition of wound repair by anti-fibrinogen.

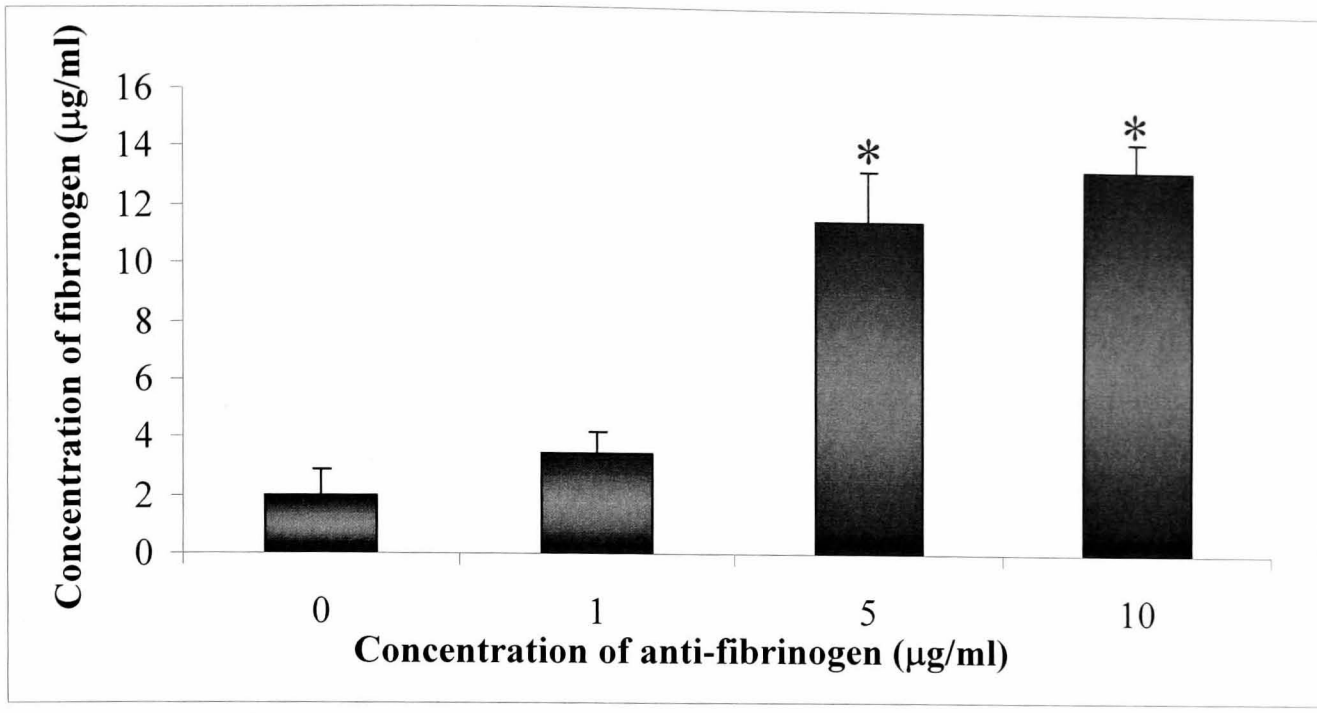

Figure 4.6. Effect of anti-fibrinogen on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells 13 hours *post*-wounding. Data represent mean ± SEM (*n=3*). * Indicates P<0.05 compared to no antibody control.

At 13 hours, the addition of 5 µg/ml anti-fibrinogen generated a significant increase in the concentration of fibrinogen (11.49 ± 1.69 µg/ml) in the cell culture supernatants compared to the no antibody control (1.96 ± 0.88 µg/ml). The concentration of fibrinogen in culture supernatants was further increased to 13.33 ± 0.95 µg/ml with the addition of 10 µg/ml anti-fibrinogen.

4.4.3.3. Concentration of FXIIIA in cell culture supernatants

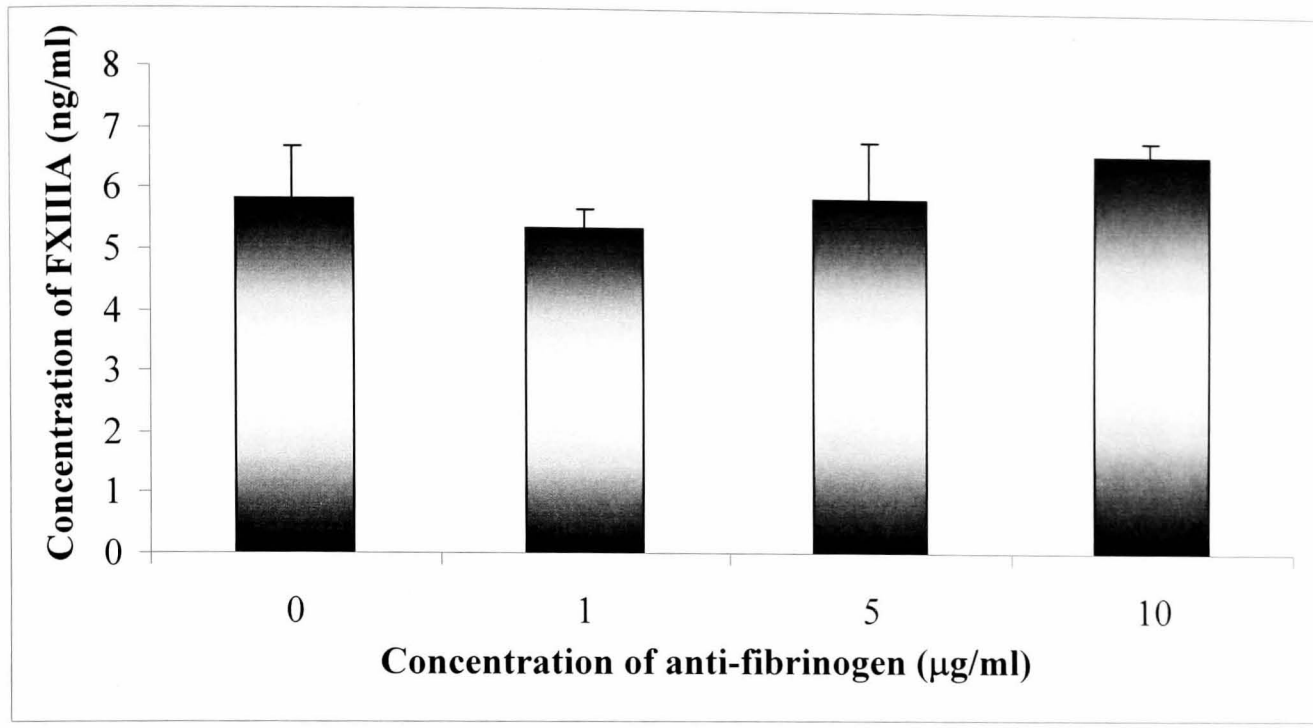

Figure 4.7. Effect of anti-fibrinogen on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells at 13 hours. Data represent mean ± SEM (*n=3*).

The concentration of FXIIIA in wounded cell monolayers was 5.83 ± 0.83 ng/ml and although anti-fibrinogen inhibited wound repair, there was no significant effect on the levels of this coagulation factor.

4.4.4. Role of FXIII in 16HBE 14o$^-$ cells

4.4.4.1. Wound repair

Since FXIII is known to play an important role in the cross-linking of fibrinogen to form a stable fibrin clot to support wound repair and its concentration in cell culture supernatants was shown to increase depending on the extent of wounding (*section 3.4.2.2*), the effect of an antibody to FXIIIA was investigated in this model of 16HBE 14o$^-$ wound repair.

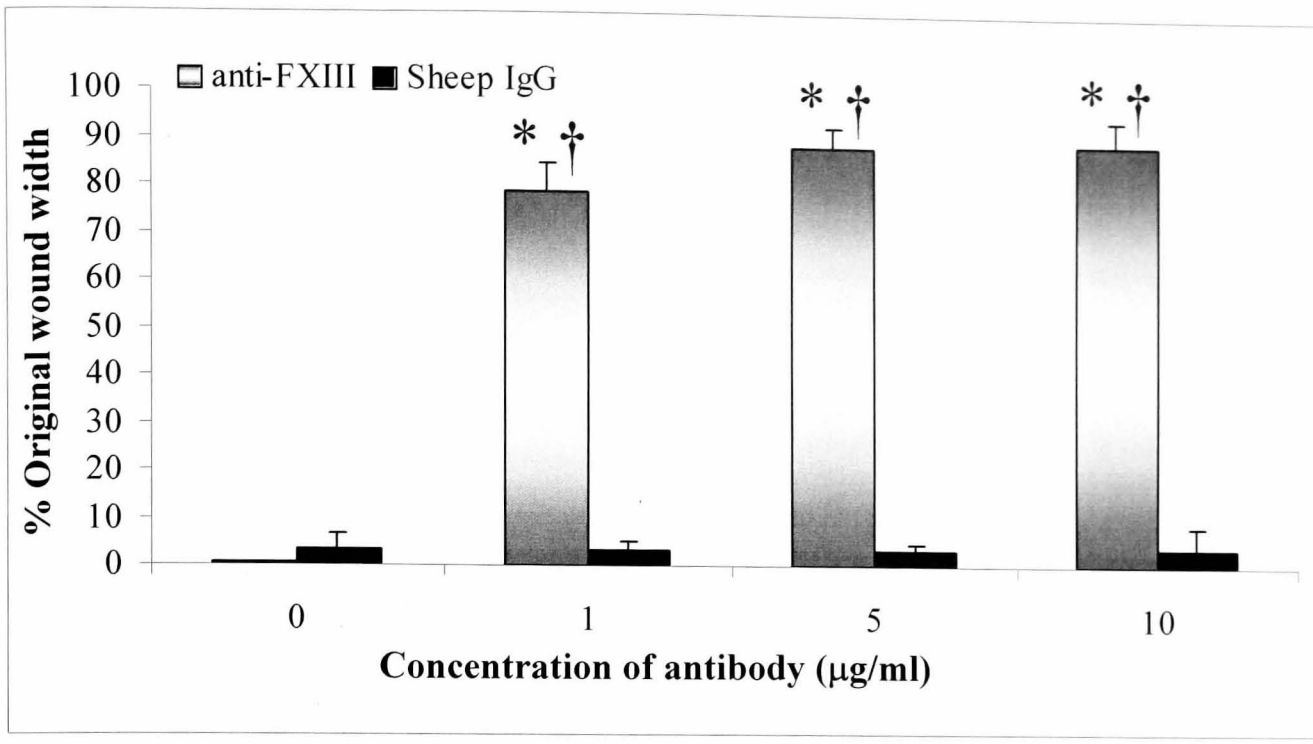

Figure 4.8. Effect of anti-FXIIIA on repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.001, compared to no-antibody control and † indicates P<0.001 compared to non-immune sheep immunoglobulin.

Repair in the untreated cell layers was almost complete with 0.42 ± 0.21 % original wound width remaining at 13 hours of culture. This effect was significantly inhibited with the addition of 1 μg/ml anti-FXIIIA and the % original wound width was 78.49 ± 6.06 %. This effect was also induced by the addition of 5 μg/ml anti-FXIIIA (88.22 ± 4.54 %) and 10 μg/ml anti-FXIIIA (89.53 ± 5.01 %). There was no effect of the non-immune sheep immunoglobulin on bronchial epithelial repair.

4.4.4.2. Concentration of fibrinogen in cell culture supernatants

Because FXIIIA is involved in the final step of fibrin formation, it was of interest to investigate the levels of fibrinogen and FXIIIA in cell culture supernatants 13 hours following inhibition of wound repair by anti-FXIIIA.

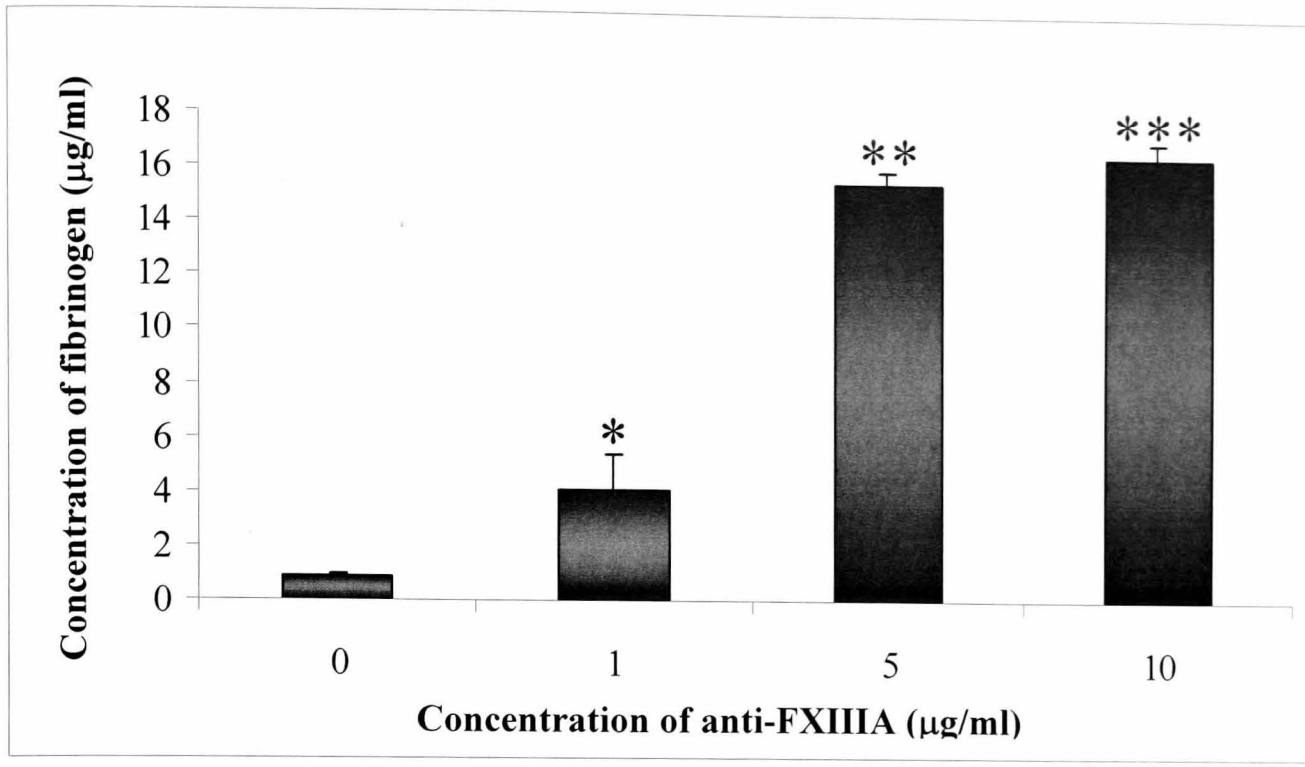

Figure 4.9. Effect of anti-FXIIIA on the concentration of fibrinogen in cell culture supernatants from 16HBE 14o$^-$ cells at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.05, ** indicates P<0.01 and *** indicates P<0.005 compared to no antibody control.

Inhibition of the function of FXIIIA with a neutralising antibody increased the amount of soluble fibrinogen detected in cell culture supernatants of 16HBE 14o$^-$ cells. The addition of 1 µg/ml anti-FXIIIA induced a four-fold increase in the concentration of fibrinogen in cell culture supernatants from 0.84 ± 0.12 µg/ml in the absence of antibody to 4.10 ± 1.43 µg/ml. Supernatant concentration of fibrinogen was further significantly increased to 15.40 ± 0.53 µg/ml with the addition of 5 µg/ml and 16.46 ± 0.62 µg/ml with the addition of 10 µg/ml anti-FXIIIA.

4.4.4.3. Concentration of FXIIIA in cell culture supernatants

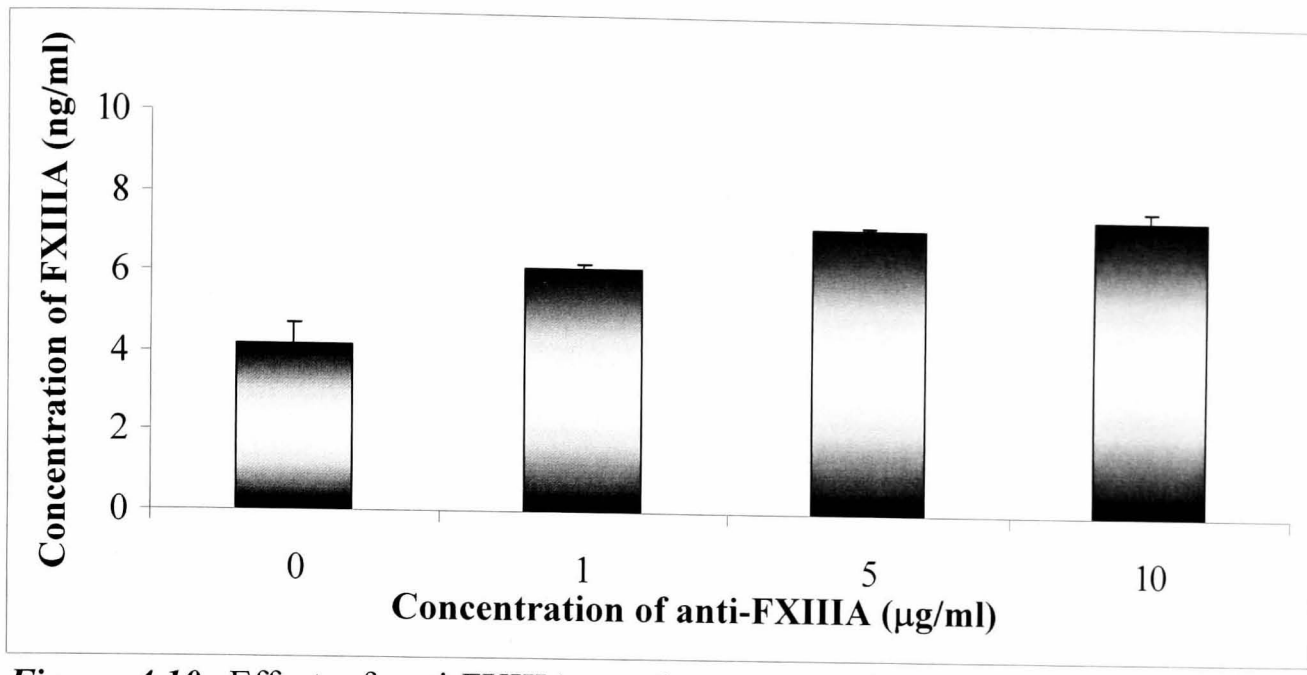

Figure 4.10. Effect of anti-FXIIIA on the concentration of FXIIIA in cell culture supernatants from 16HBE 14o$^-$ cells at 13 hours. Data represent mean ± SEM (*n=3*).

FXIIIA concentration in wounded cell monolayers was 4.19 ± 0.53 ng/ml. The addition of anti-FXIIIA had no significant effect on the concentration of FXIIIA in cell culture supernatants from 16HBE 14o$^-$ cells; however, at 10 µg/ml anti-FXIIIA, the concentration of FXIIIA (7.52 ± 0.20 µg/ml) was considerably higher than that of the no antibody control (4.19 ± 0.53 µg/ml), but this effect was not significant (p=0.083).

4.4.5. Role of TF in NHBE cells

Since anti-TF inhibited wound repair in the 16HBE 14o$^-$ cell line, it was of interest to investigate its effect in wound repair of primary NHBE cells. It must be noted that NHBE cells did not adopt the 'cobblestone' appearance in culture and did not form the tight junctions, which are evident in the 16HBE 14o$^-$ cell line.

Wound repair of NHBE cells was monitored over 20 hours. This time-point was chosen, as repair of NHBE cells is considerably slower than that of 16HBE 14o$^-$ cells, most likely due to the fact that NHBE cells do not possess tight junctions in submerged culture and may therefore lack the required cell signalling to initiate repair.

4.4.5.1. Wound repair

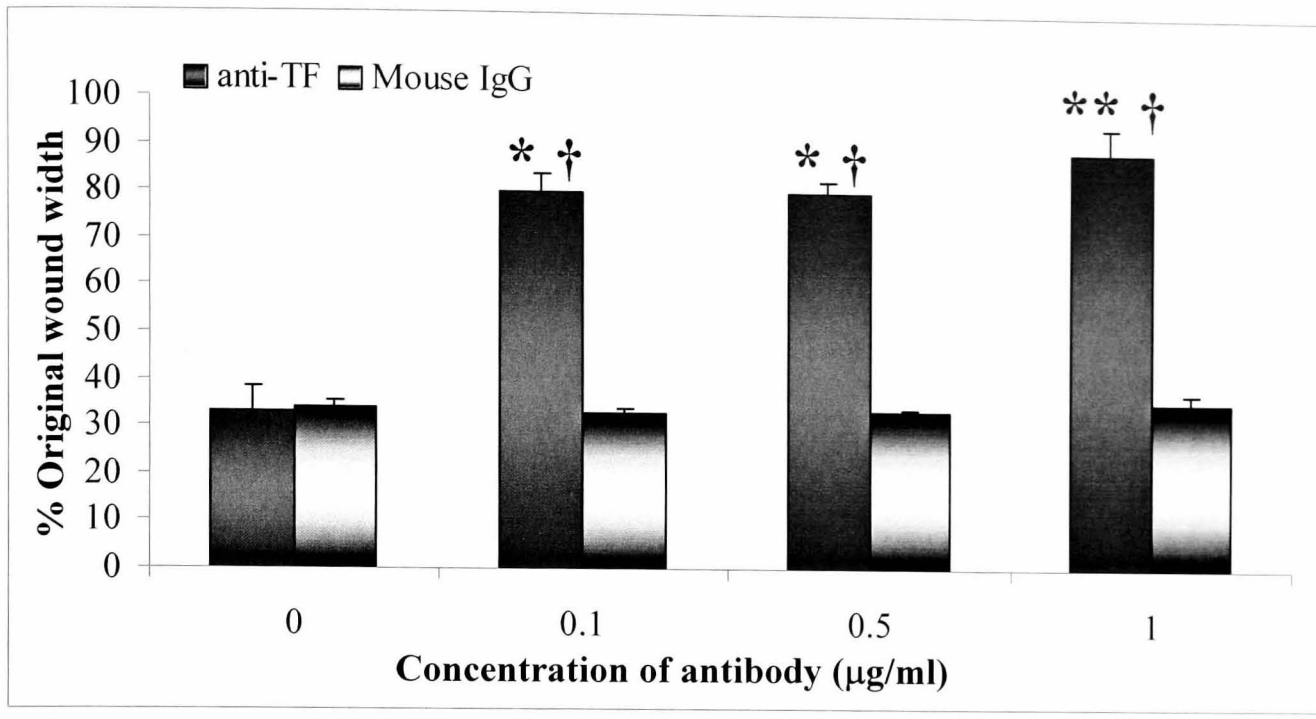

Figure 4.11. Effect of anti-TF and non-immune mouse IgG on bronchial epithelial repair of NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*). * Indicates $P<0.05$, ** indicates $P<0.001$ compared to no-antibody control and † indicates $P<0.05$ compared to non-immune mouse IgG.

The time-course for NHBE wound repair was different to that seen in 16HBE 14o$^-$ cells. After 20 hours, the wound had not completely closed but remained open at 33.16 ± 5.12 % of the original wound width. Wound repair of NHBE cells was significantly impaired with the addition of 0.1 μg/ml anti-TF, i.e. 79.88 ± 4.25 % original wound width compared to 33.16 ± 5.12 % in the absence of antibody. Inhibition of wound repair was maintained with the addition of 0.5 μg/ml anti-TF (80.30 ± 2.10 %) and 1 μg/ml anti-TF (88.86 ± 5.14 %). There was no effect of the non-immune mouse immunoglobulin on bronchial epithelial repair.

4.4.5.2. Concentration of fibrinogen in cell culture supernatants

Since TF is important in the initiation of the coagulation cascade, leading to fibrin formation and anti-TF significantly decreased wound repair in NHBE cells, it was of interest to investigate the levels of fibrinogen and FXIIIA in cell culture supernatants at 20 hours following stimulation with anti-TF.

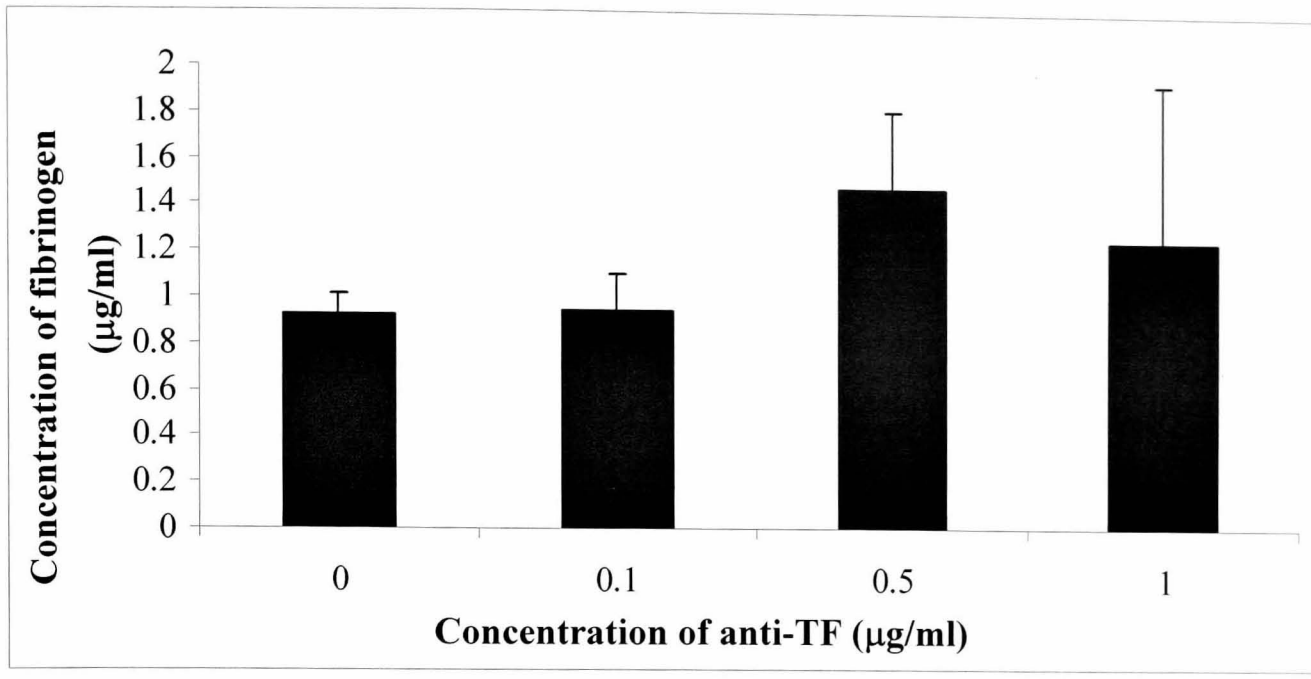

Figure 4.12. Effect of anti-TF on the concentration of fibrinogen in cell culture supernatants of NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*).

Although anti-TF significantly decreased wound repair, there was no significant effect on the concentration of fibrinogen in cell culture supernatants of NHBE cells.

4.4.5.3. Concentration of FXIIIA in cell culture supernatants

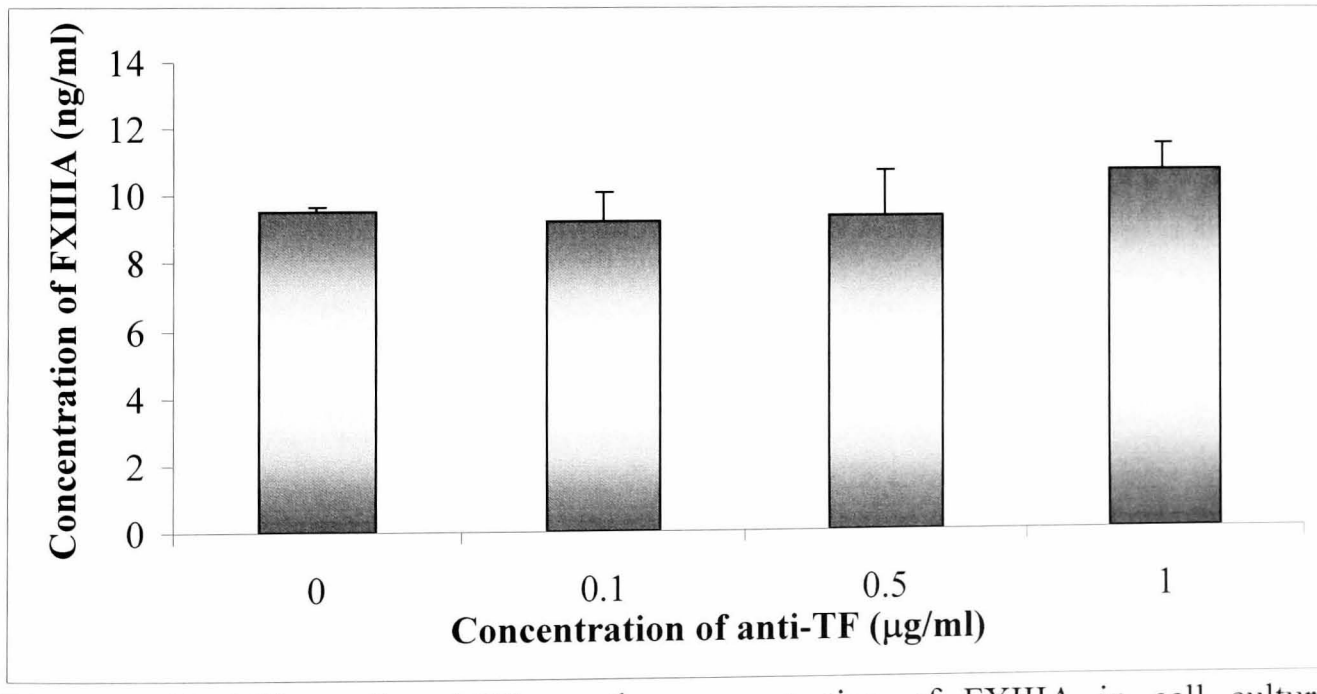

Figure 4.13. Effect of anti-TF on the concentration of FXIIIA in cell culture supernatants of NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*).

FXIIIA concentration in cell culture supernatants of wounded monolayers 20 hours *post*-wounding were 9.53 ± 0.14 ng/ml. Analysis of cell culture supernatants from

NHBE cells exposed to anti-TF revealed that there was no significant effect of the antibody on the concentration of FXIIIA, as seen in 16HBE 14o$^-$ cells.

4.4.6. Role of fibrinogen in NHBE cells

4.4.6.1. Wound repair

Fibrinogen is the precursor for fibrin, which has a role in bronchial epithelial repair. Anti-fibrinogen was shown to inhibit wound repair in the 16HBE 14o$^-$ cell line; therefore, its effects were investigated in primary NHBE cells.

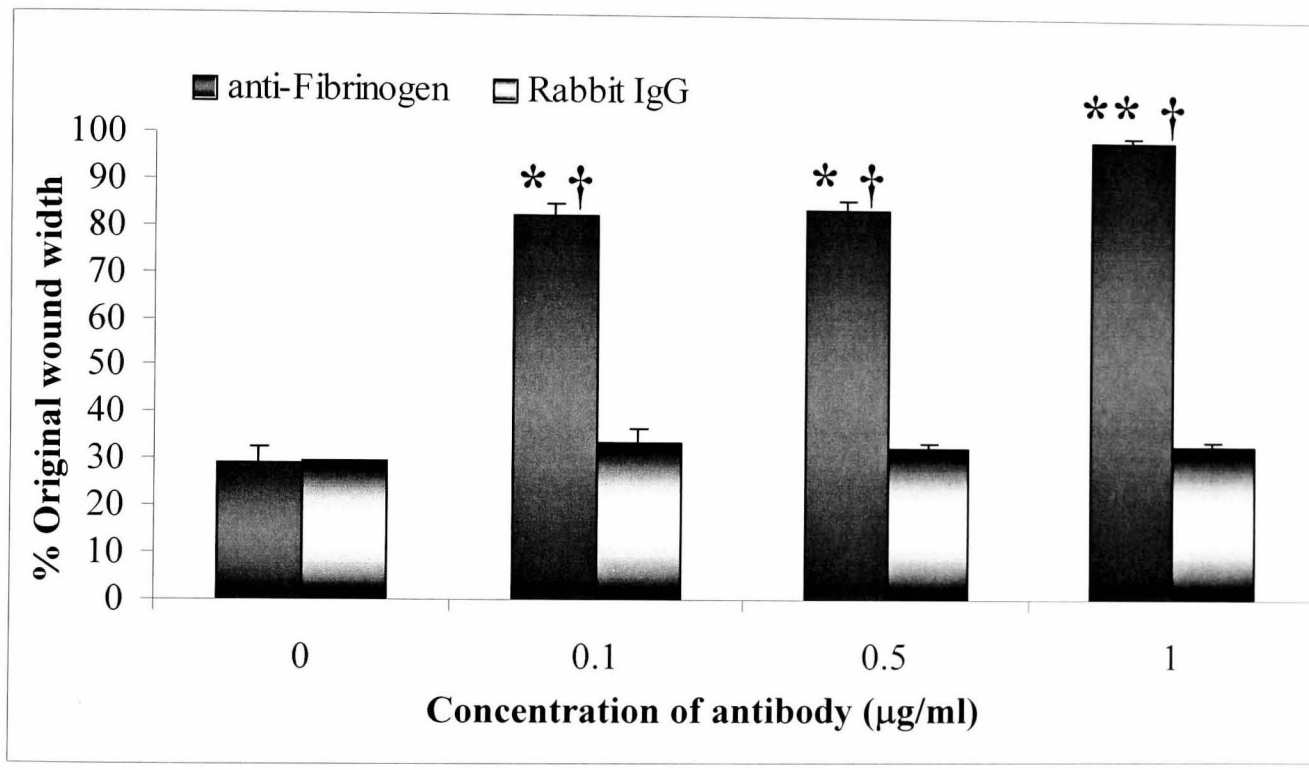

Figure 4.14. Effect of anti-fibrinogen and non-immune rabbit IgG on bronchial epithelial repair of NHBE cells at 20 hours. Data represents mean ± SEM (*n=3*). * Indicates P<0.01, ** indicates P<0.001 compared to no antibody control and † indicates P<0.05 compared to non-immune rabbit IgG.

The addition of 0.1 μg/ml anti-fibrinogen significantly reduced wound repair to 82.64 ± 2.21 % original wound width at 20 hours compared to 28.98 ± 3.67 % in the absence of antibody. This effect was maintained with the addition of 0.5 μg/ml anti-fibrinogen (83.90 ± 2.21 %) and 1 μg/ml anti-fibrinogen, whereby wound repair (99.02 ± 1.11 % original wound width) was almost completely inhibited. There was no effect of the non-immune rabbit immunoglobulin on bronchial epithelial repair.

4.4.6.2. Concentration of fibrinogen in cell culture supernatants

Since fibrinogen is involved in the final step of fibrin formation and anti-fibrinogen significantly decreased wound repair in NHBE cells, it was of interest to investigate the levels of fibrinogen and FXIIIA in cell culture supernatants at 20 hours following stimulation with anti-fibrinogen.

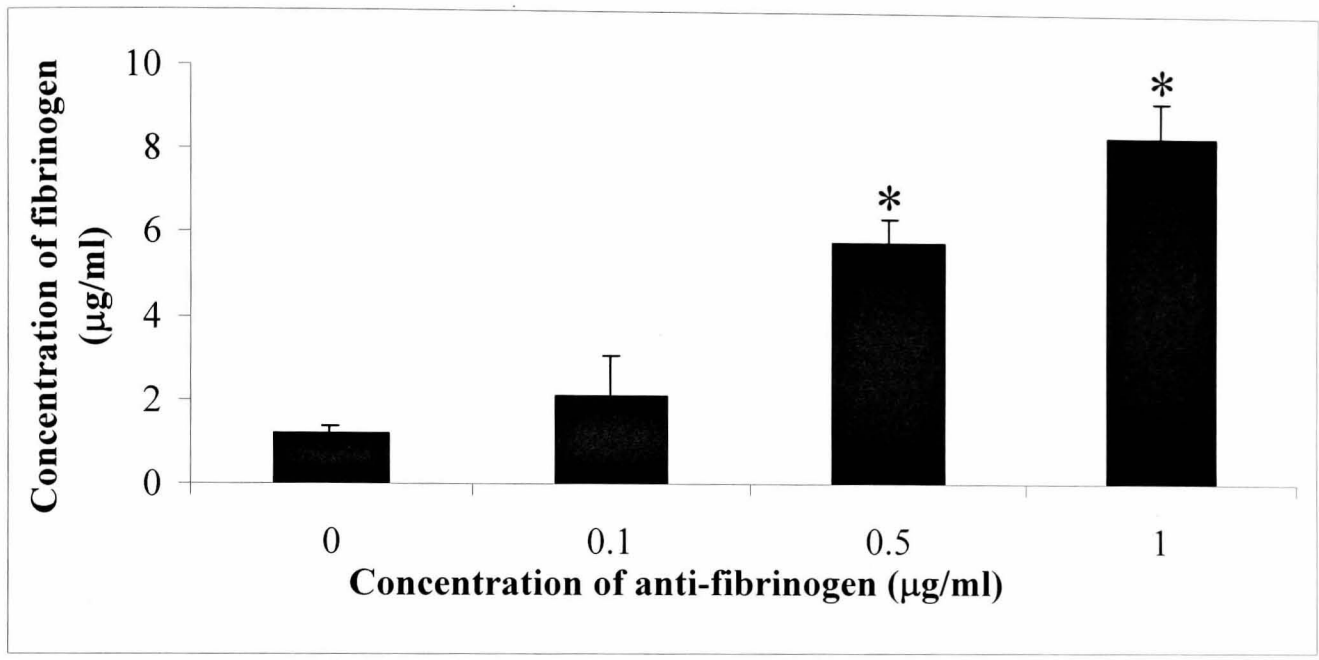

Figure 4.15. Effect of anti-fibrinogen on the concentration of fibrinogen in cell culture supernatants from NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*) * Indicates P<0.005 compared to no antibody control.

Anti-fibrinogen did however stimulate a significant increase in the concentration of fibrinogen in cell culture supernatants with the addition of 0.5 µg/ml (5.82 ± 0.52 µg/ml fibrinogen) and 1 µg/ml (8.35 ± 0.86 µg/ml fibrinogen) compared to the no antibody control (1.21 ± 0.15 µg/ml fibrinogen).

4.4.6.3. Concentration of FXIIIA in cell culture supernatants

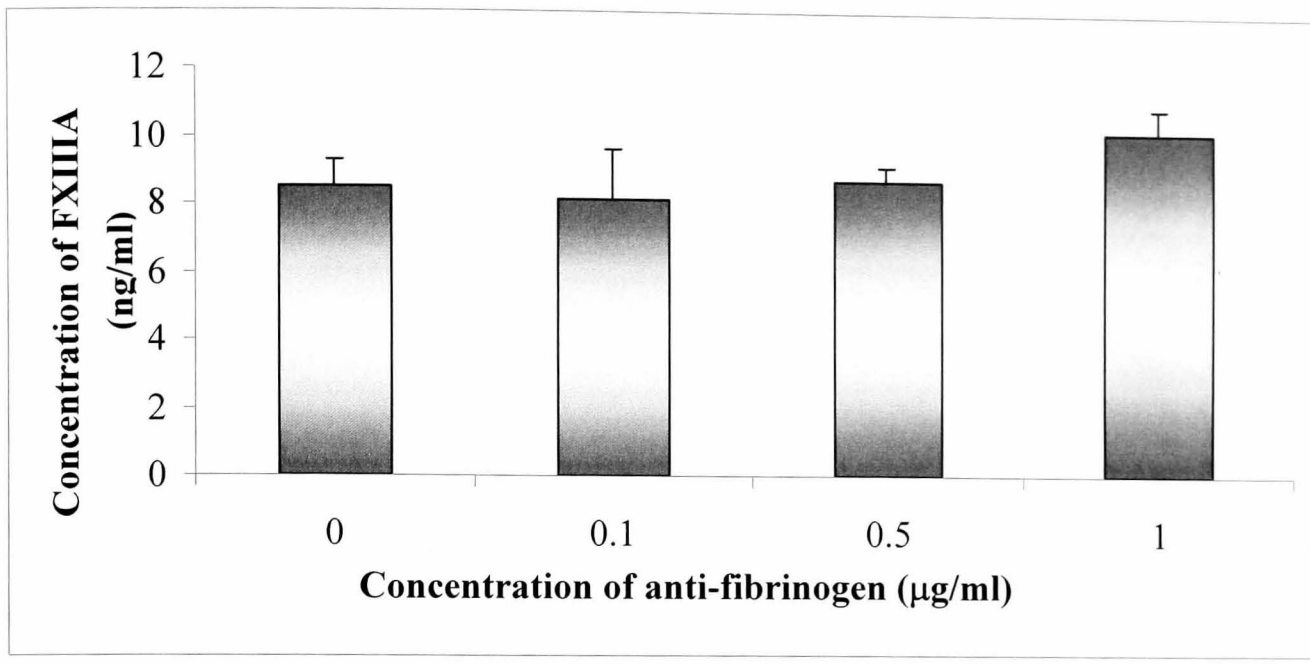

Figure 4.16. Effect of anti-fibrinogen on the concentration of FXIIIA in cell culture supernatants from NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*).

Analysis of cell culture supernatants at 20 hours revealed that there was no effect of anti-fibrinogen on the concentration of FXIIIA compared to the untreated cells despite inhibition of wound repair by anti-fibrinogen.

4.4.7. Role of FXIII in NHBE cells

4.4.7.1. Wound repair

Since anti-FXIIIA was shown to significantly inhibit wound repair in 16HBE 14o$^-$ cells, it was of interest to investigate its effects in a model of primary NHBE wound repair.

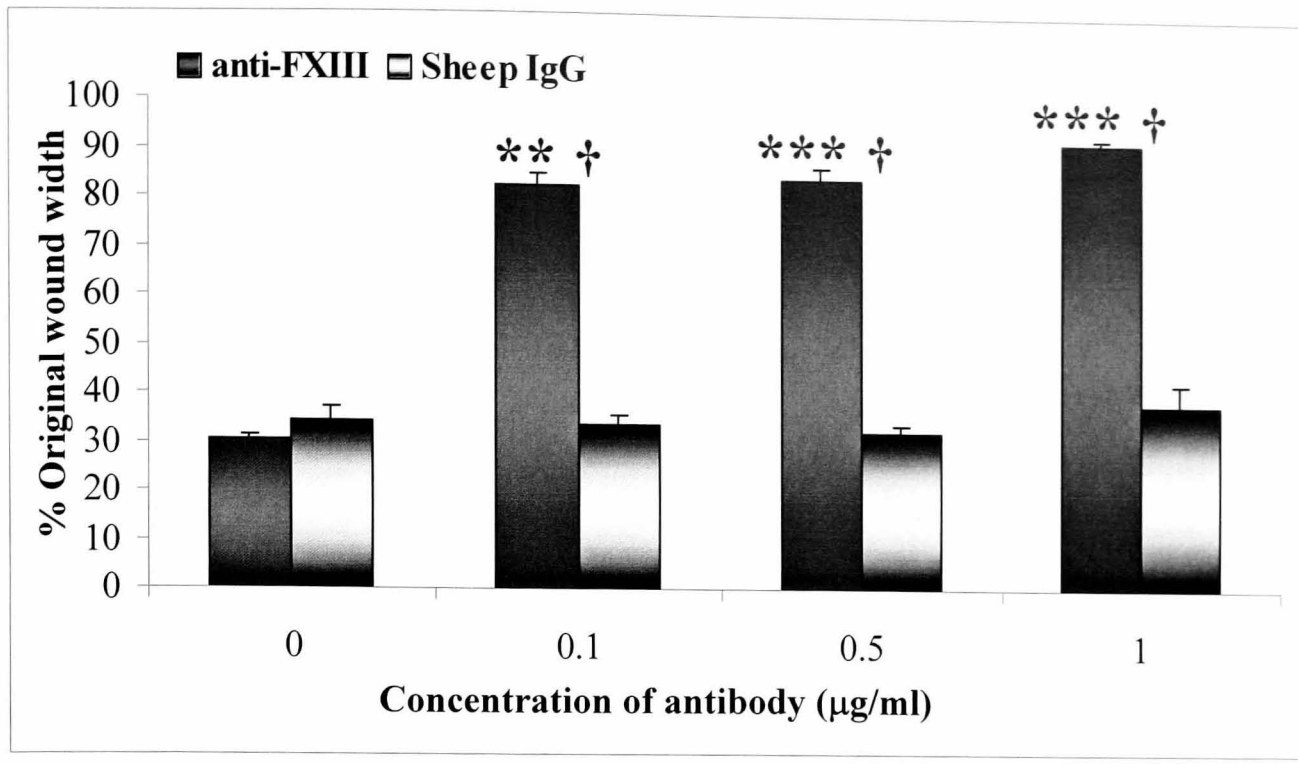

Figure 4.17. Effect of anti-FXIIIA and non-immune sheep IgG on bronchial epithelial repair of NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*). ** Indicates P<0.01 *** indicates P<0.001 compared to no antibody control and † indicates P<0.05 compared to non-immune sheep IgG.

Anti-FXIIIA significantly reduced bronchial epithelial repair to 82.39 ± 2.39 % original wound width with the addition of 0.1 µg/ml anti-FXIIIA compared to the no antibody control (30.01 ± 1.34 %). This effect was maintained with the addition of 0.5 µg/ml anti-FXIIIA (83.82 ± 2.49 %) and 1 µg/ml anti-FXIIIA (91.79 ± 1.07 %). There was no effect of the non-immune sheep immunoglobulin.

4.4.7.2. Concentration of fibrinogen in cell culture supernatants

FXIII is important in the cross-linking of fibrinogen to form a stable fibrin clot. Since anti-FXIIIA was shown to significantly decrease wound repair of NHBE cells, it was of interest to determine the levels of fibrinogen and FXIIIA in cell culture supernatants at 20 hours following stimulation with anti-FXIIIA.

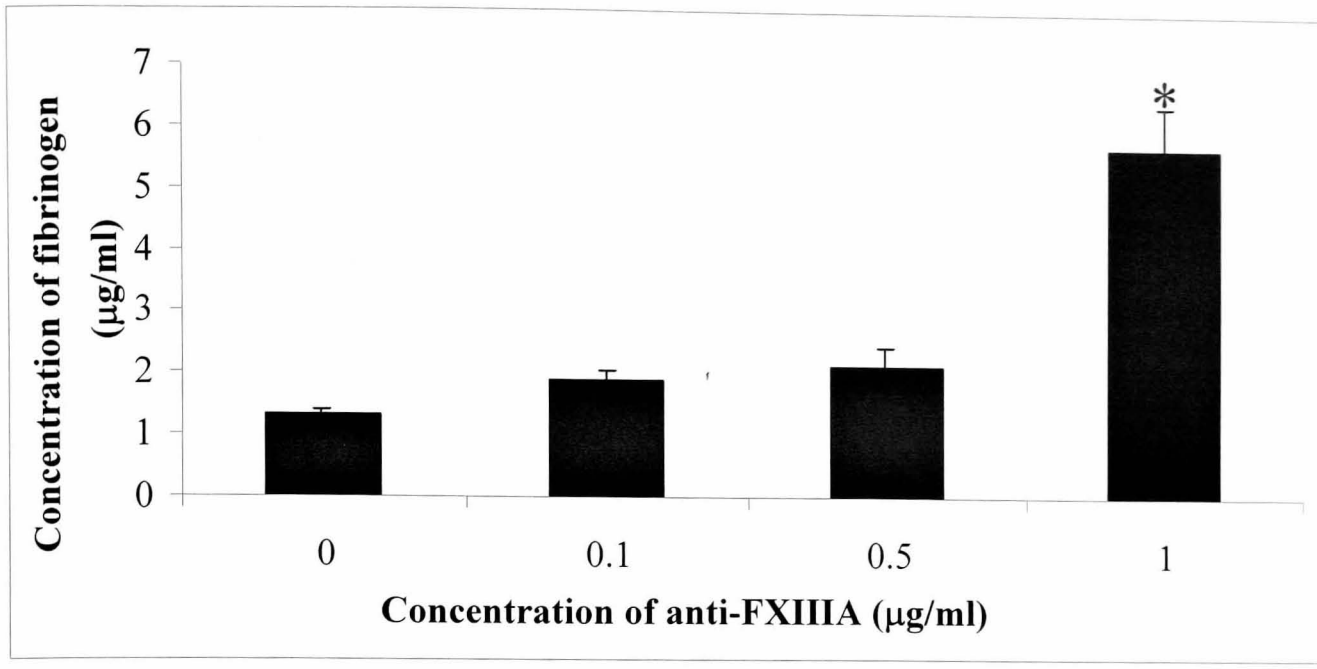

Figure 4.18. Effect of anti-FXIIIA on the concentration of fibrinogen in cell culture supernatants from NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.05 compared to no antibody control.

Analysis of cell culture supernatants at 20 hours revealed that the addition of 1 µg/ml anti-FXIIIA resulted in a significant 5-fold increase in the concentration of fibrinogen (5.68 ± 1.45 µg/ml) compared to 1.32 ± 0.11 µg/ml fibrinogen in the no antibody control.

4.4.7.3. Concentration of FXIIIA in cell culture supernatants

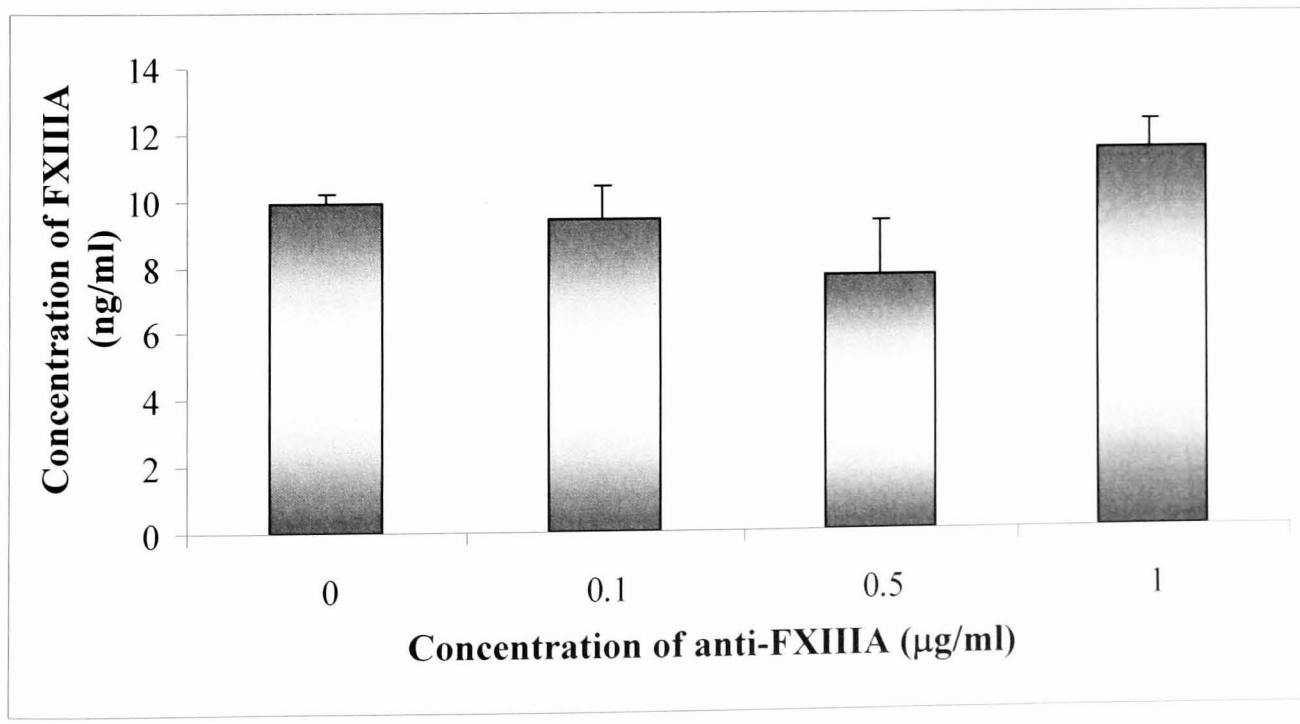

Figure 4.19. Effect of anti-FXIIIA on the concentration of FXIIIA in cell culture supernatants from NHBE cells at 20 hours. Data represent mean ± SEM (*n=3*).

Despite inhibition of wound repair by anti-FXIIIA, there was no effect on the concentration of FXIIIA in cell culture supernatants at 20 hours compared to the no antibody control.

4.4.8. Role of FXa

4.4.8.1. Exogenous FXa

The serine protease FXa plays a central role in the coagulation cascade, linking the extrinsic and intrinsic pathways by catalysing the conversion of prothrombin to thrombin, which subsequently leads to the formation of fibrin. It is also an activator of PAR-1 and PAR-2. Thus, it was of interest to investigate the role of FXa. Since FXa is involved in fibrin formation, it was expected that an increase in coagulation by the addition of FXa would result in an increase in wound repair.

The concentration range of FXa (0-200 nM) was employed as it corresponded to that which was previously reported and is physiologically relevant as plasma contains 130 nM FXa.

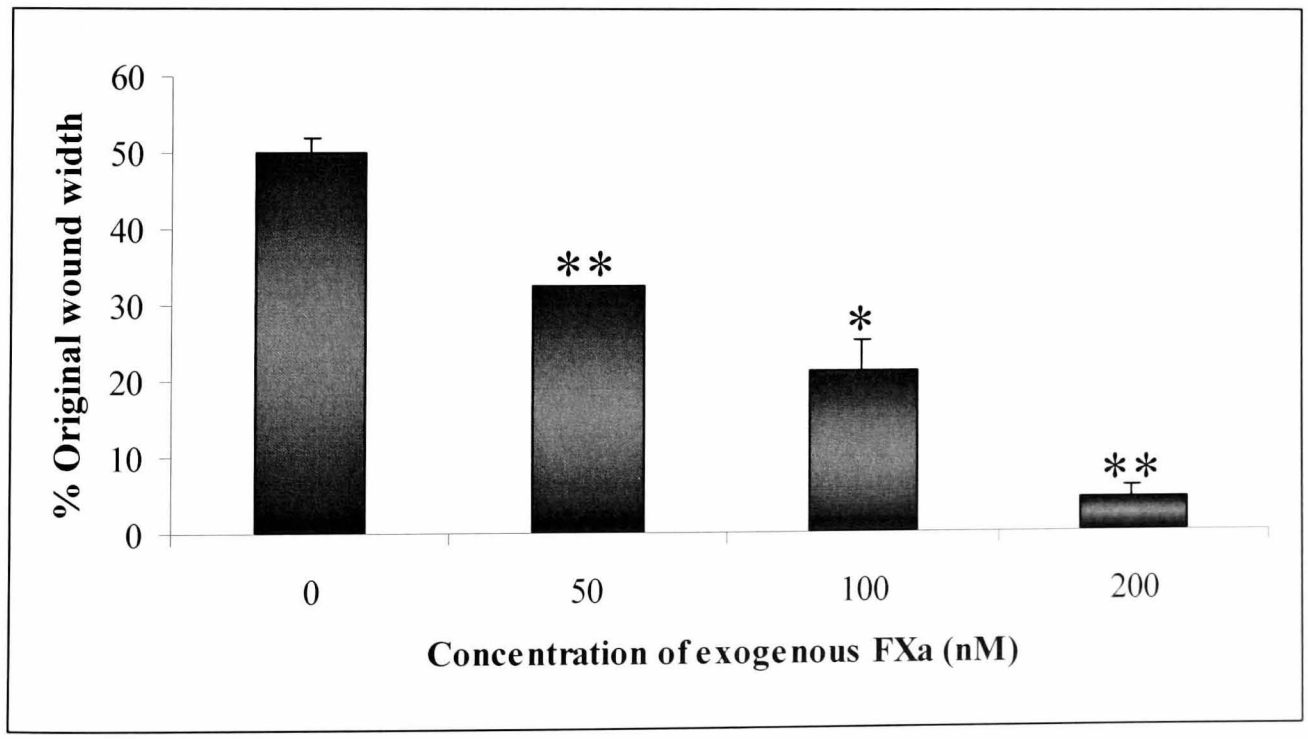

Figure 4.20. Effect of exogenous FXa on wound repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM ($n=3$). * Indicates $P<0.0005$ and ** indicates $P<0.0001$ compared to no drug control.

The addition of exogenous FXa to 16HBE 14o$^-$ cells significantly enhanced wound repair in a dose-dependent manner. In the presence of 50 nM FXa, the % original wound width was reduced from 50.04 ± 1.83 % in the untreated cell cultures to 32.51 ± 0.12 %. Wound repair was further enhanced in the presence of 100 nM FXa (21.39 ± 4.09 %) and 200 nM, whereby the % original wound width was reduced to 4.42 ± 1.63 %.

4.4.8.2. Endogenous FXa

Since the addition of FXa enhanced bronchial epithelial repair, the role of endogenous FXa in wound repair was investigated with the use of a selective FXa inhibitor encoded UK-220,047-01, also termed DX9065A, a Daiichi compound provided by Pfizer Global R&D.

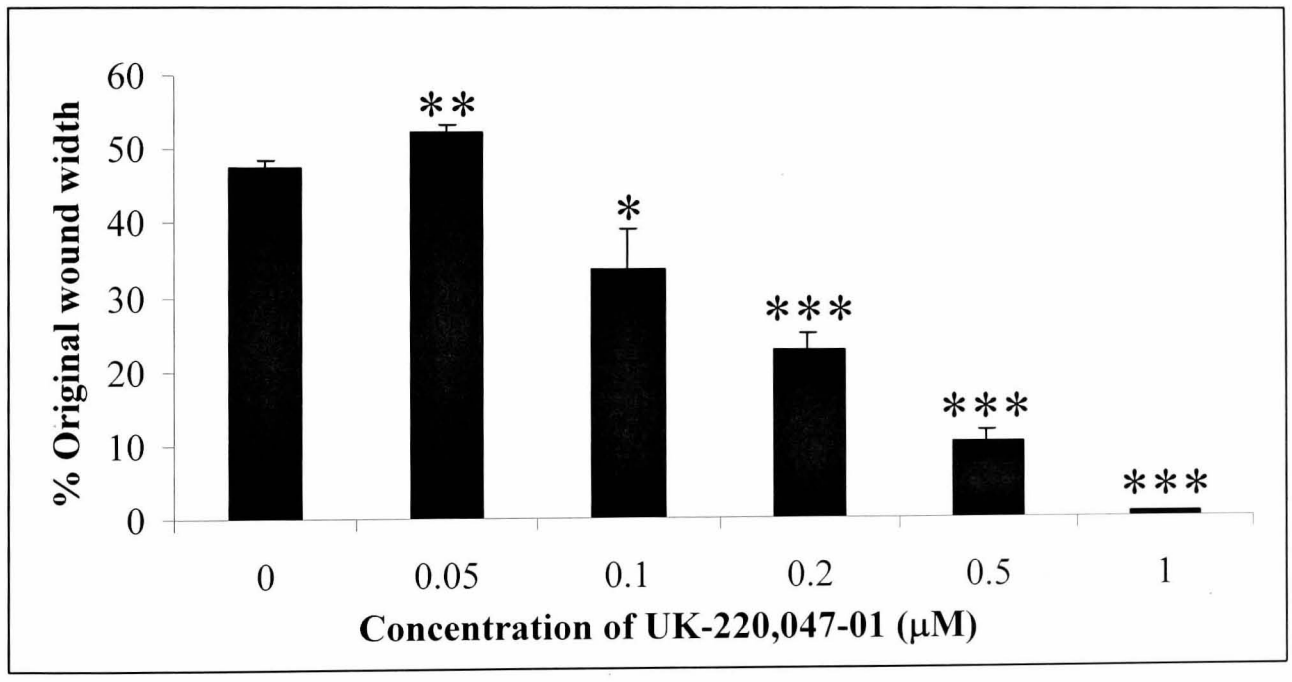

Figure 4.21. Effect of the FXa inhibitor, UK-220,047-01 on wound repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.05, ** indicates P<0.01 and *** indicates P<0.0001 compared to no drug control.

Wound repair was significantly reduced, but only with the addition of the lowest concentration of 0.05 μM UK-220,047-01, whereby the % original wound width was 52.23 ± 1.09 % compared to 47.42 ± 1.15 % in the untreated cell cultures.

However, contrary to expectations, the addition of the FXa inhibitor UK-220,047-01 in the concentration range of 0.1-1 μM *enhanced* wound repair in a dose-responsive

manner suggesting an alternative role for endogenous FXa. Wound widths following the addition of: 0.1 μM UK-220,047-01 = 33.63 ± 5.62 %; 0.2 μM UK-220,047-01 = 22.73 ± 2.32 %; 0.5 μM UK-220,047-01 = 10.20 ± 1.75 % and 1 μM UK-220,047-01 = 0.51 ± 0.08 %.

Because of this unexpected finding, a second more potent and selective FXa inhibitor encoded PD-031 (Pfizer Global R&D) was used to confirm the role of endogenous FXa.

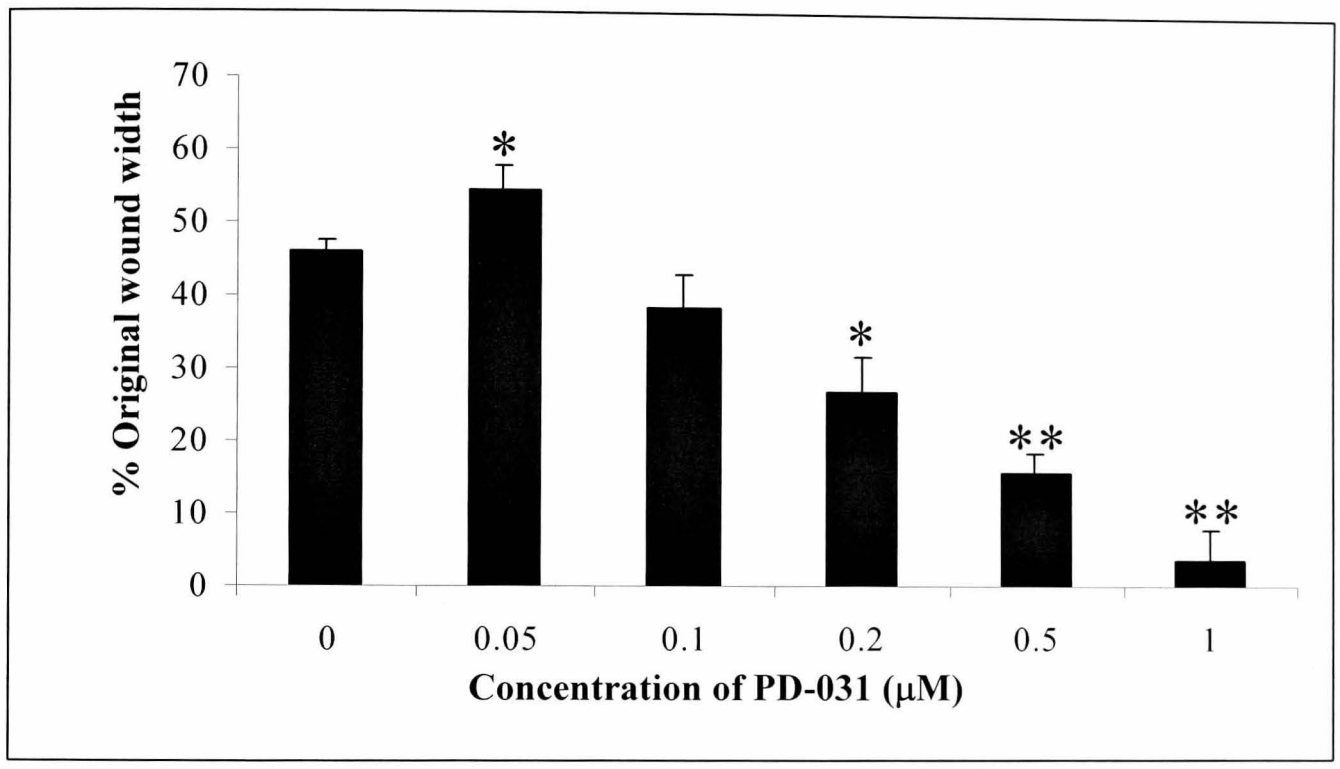

Figure 4.22. Effect of the FXa inhibitor, PD-031 on wound repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.05 and ** indicates P<0.0001 compared to no drug control.

Similarly, the lowest concentration of PD-031 significantly reduced wound repair (54.43 ± 3.50 % original wound width) compared to 45.95 ± 1.62 % in the untreated cell cultures. However, higher concentrations of PD-031 *stimulated* wound repair in a dose-responsive manner. Wound widths following the addition of: 0.1 μM PD-031 = 38.62 ± 4.31 %; 0.2 μM PD-031 = 26.85 ± 4.96 %; 0.5 μM PD-031 = 15.89 ± 2.70 % and 1 μM PD-031 = 3.55 ± 4.21 %.

4.4.9. Role of endogenous thrombin

Since the FXa inhibitor unexpectedly enhanced wound repair, it was of interest to investigate the role of endogenous thrombin in wound repair with the use of a thrombin inhibitor encoded UK-156,406, which was provided by Pfizer Global R&D.

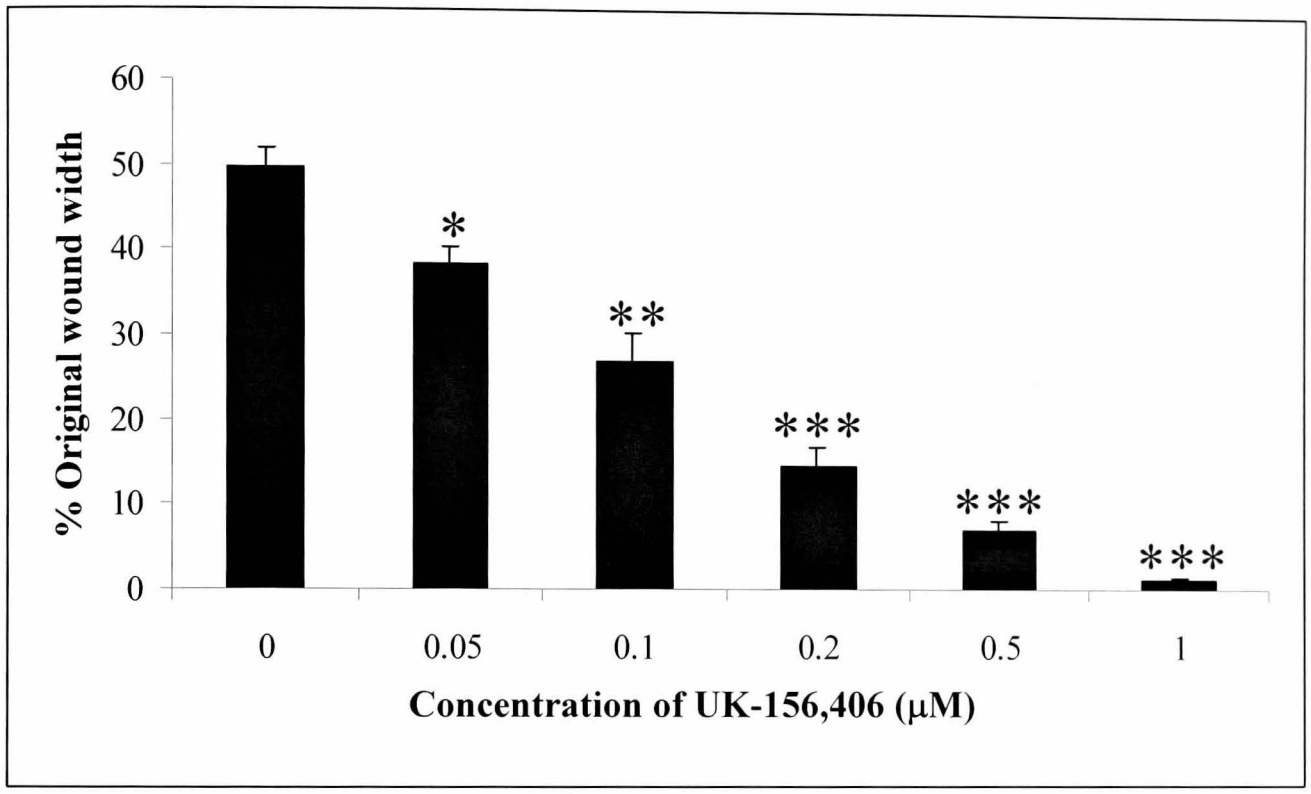

Figure 4.23. Effect of the thrombin inhibitor, UK156,406 on wound repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.005, ** indicates P<0.001 and *** indicates P<0001 compared to no drug control.

The % original wound width was significantly reduced and therefore wound repair enhanced in a dose-responsive manner with the addition of: 0.05 μM UK-156,406 = 38.42 ± 1.83 %; 0.1 μM UK-156,406 = 27.01 ± 3.27 %; 0.2 μM UK-156,406 = 14.59 ± 2.11 %; 0.5 μM UK-156,406 = 7.01 ± 1.07 % and 1 μM UK-156,406 = 1.24 ± 0.28 % compared to the no drug control (49.52 ± 2.41 %) at 13 hours.

4.4.10. Involvement of the COX pathway in wound repair

Since both FXa inhibitors: UK-220,047-01 and PD-031 enhanced wound repair (*section 4.4.8.2: figures 4.21 and 4.22* respectively), the role of endogenous FXa in the coagulation cascade was questioned. Reports have demonstrated that cellular signalling by FXa in tissue culture has been shown to have effects that are independent from

thrombin generation and that are considered to be pro-inflammatory (Feistritzer *et al.*, 2005). Furthermore, it has been suggested that the effects of FXa may be mediated *via* the prostaglandin receptor EP-1 to release VEGF, IL-6, IL-8, MCP-1 and CD54 (Schulman, 2003). It was hypothesised that FXa may have an alternative role in wound repair, other than its involvement in the coagulation cascade. Thus, the effects of endogenous FXa mediated *via* inflammatory cascades were investigated.

4.4.10.1. Indomethacin

A study by Savla *et al* (2001) demonstrated that PGE_2 regulates wound repair of the bronchial epithelium and that this effect is mediated *via* EP-1 and EP-4 (Savla *et al.*, 2001). PGE_2 is the end product of COX metabolism of arachidonic acid and is actively secreted by bronchial epithelial cells. Indomethacin is a COX inhibitor and therefore inhibits the production of PGE_2. Since wound repair was stimulated by the addition of two separate FXa inhibitors when added in the concentration range of 0.1-1 μM, it was of interest to investigate the effects of the selective FXa inhibitor PD-031 in the presence of indomethacin in order to investigate a role for PGE_2 in this response.

The effects of the COX inhibitor indomethacin were firstly investigated alone and then in the presence of 1 μM PD-031.

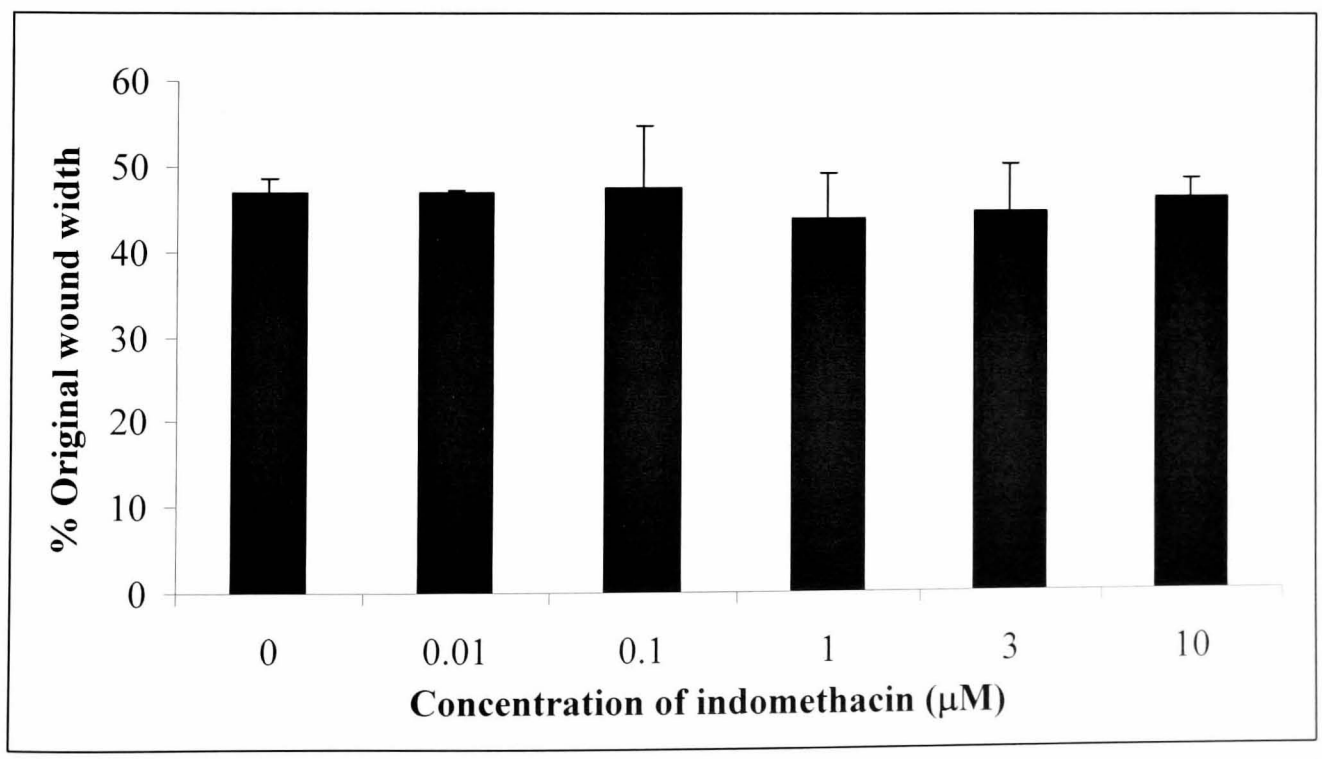

Figure 4.24. Effect of indomethacin on wound repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*).

Addition of the COX inhibitor indomethacin in the concentration range of: 0.01-10 μM had no effect on wound repair compared to the untreated cell cultures.

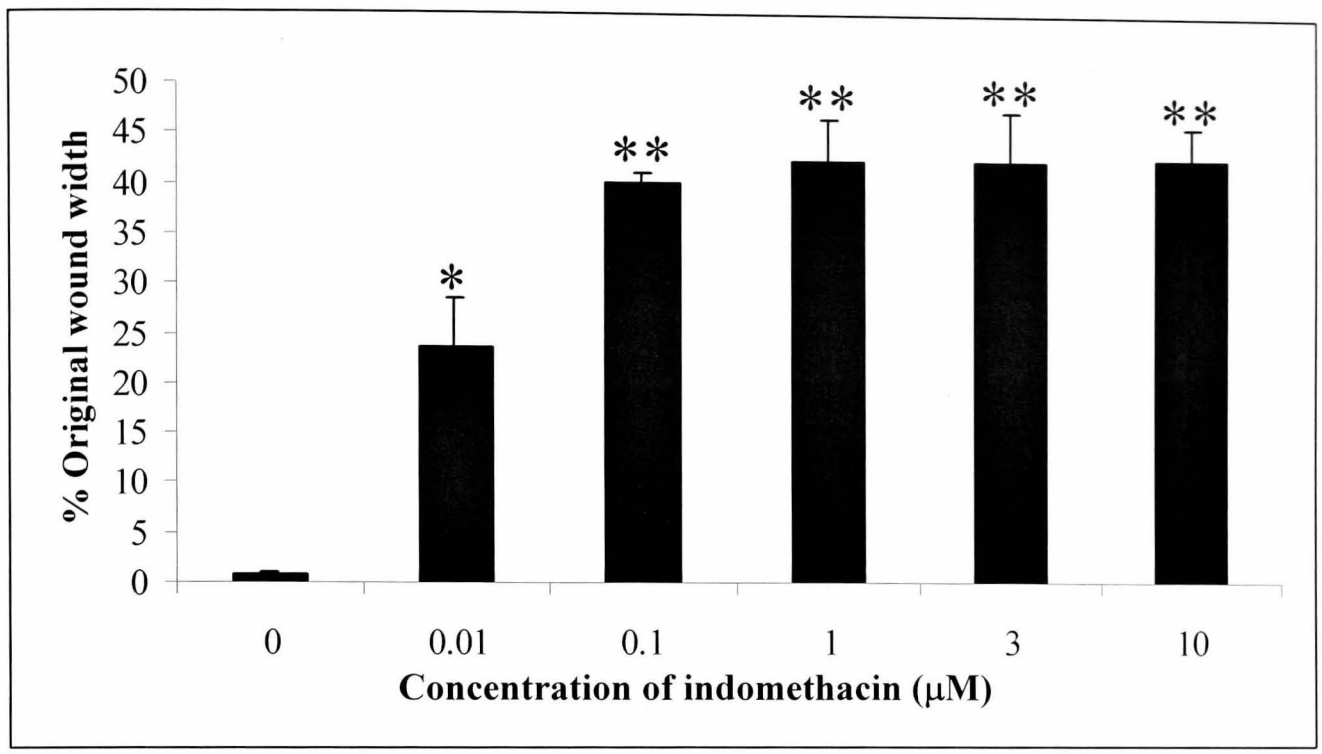

Figure 4.25. Effect of indomethacin in the presence of 1 μM PD-031 on bronchial epithelial repair of 16HBE 14o$^-$ cells at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.005 and ** indicates P<0.0001 compared to 1 μM PD-031 alone.

In order to investigate a role for PGE_2 in wound repair, the COX inhibitor indomethacin was added to cells in the presence of 1 μM PD-031, the FXa inhibitor. The addition of PD-031 alone stimulated wound repair, reducing the % original wound width to 0.81 ± 0.26 %. This effect was significantly reversed with the addition of 0.01 μM indomethacin (23.68 ± 4.89 %) and 0.1 μM indomethacin (40.13 ± 0.95 %) and a plateau was reached with the addition of 1 μM indomethacin (42.39 ± 4.10 %).

4.4.10.2. PGE_2

The significant effects of the COX inhibitor indomethacin in the presence of the FXa inhibitor PD-031 suggested that PGE_2 was involved in the repair response. Therefore, it was pertinent to investigate a role for the COX metabolite PGE_2 on wound repair. The addition of PGE_2 (0-10 μg/ml) was previously shown to enhance wound repair of 16HBE 14o$^-$ cells in the presence of 10% FBS, in a dose-responsive manner over 12 hours (Savla *et al.*, 2001). In order to confirm a role for PGE_2, its effects were investigated in the current serum-free model of 16HBE 14o$^-$ wound repair.

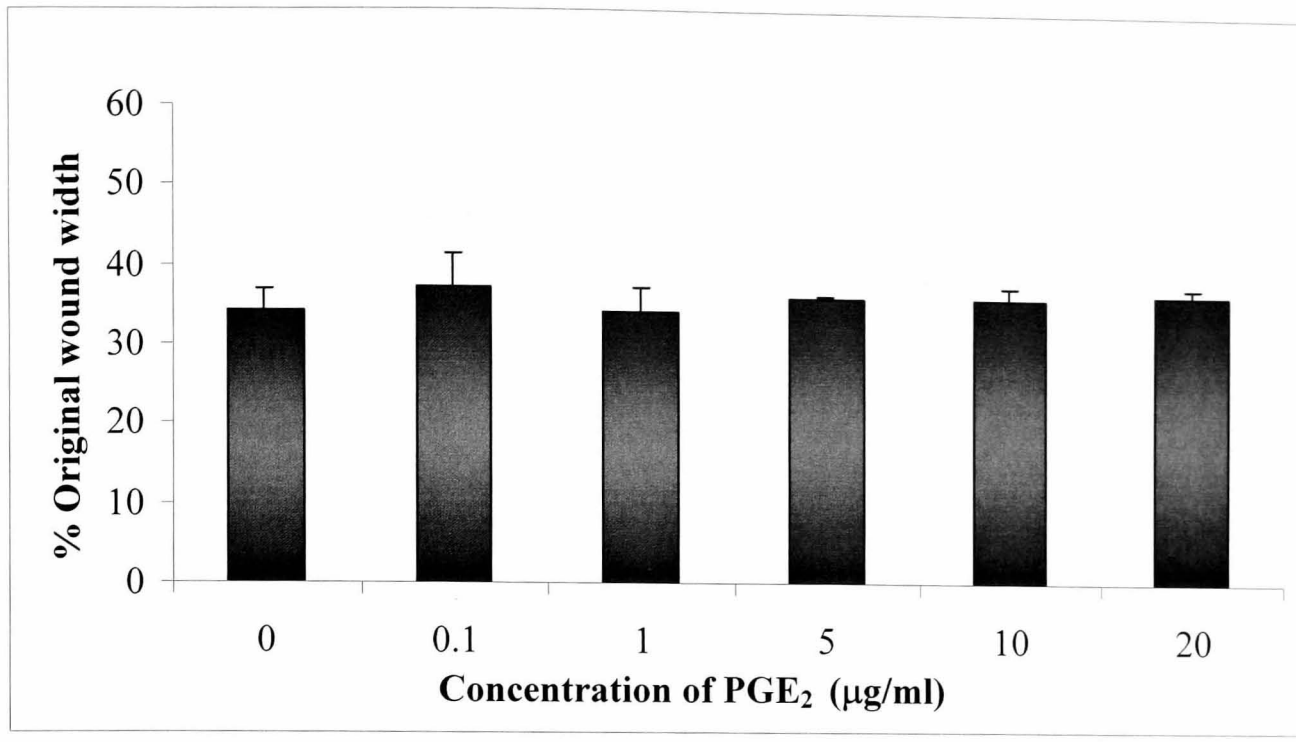

Figure 4.26. Effect of PGE_2 on wound repair of 16HBE 14o$^-$ cell layers at 13 hours. Data represent mean ± SEM (*n=3*).

However, addition of PGE_2 in the concentration range of 0-20 µg/ml had no effect on wound repair compared to the untreated cell cultures in this serum-free model.

4.4.11. Effect of neutrophil elastase on wound repair

Elastase not only degrades coagulation factors (*Chapter 3*) but also inactivates PARs. Therefore, the effect of neutrophil elastase on wound repair was investigated.

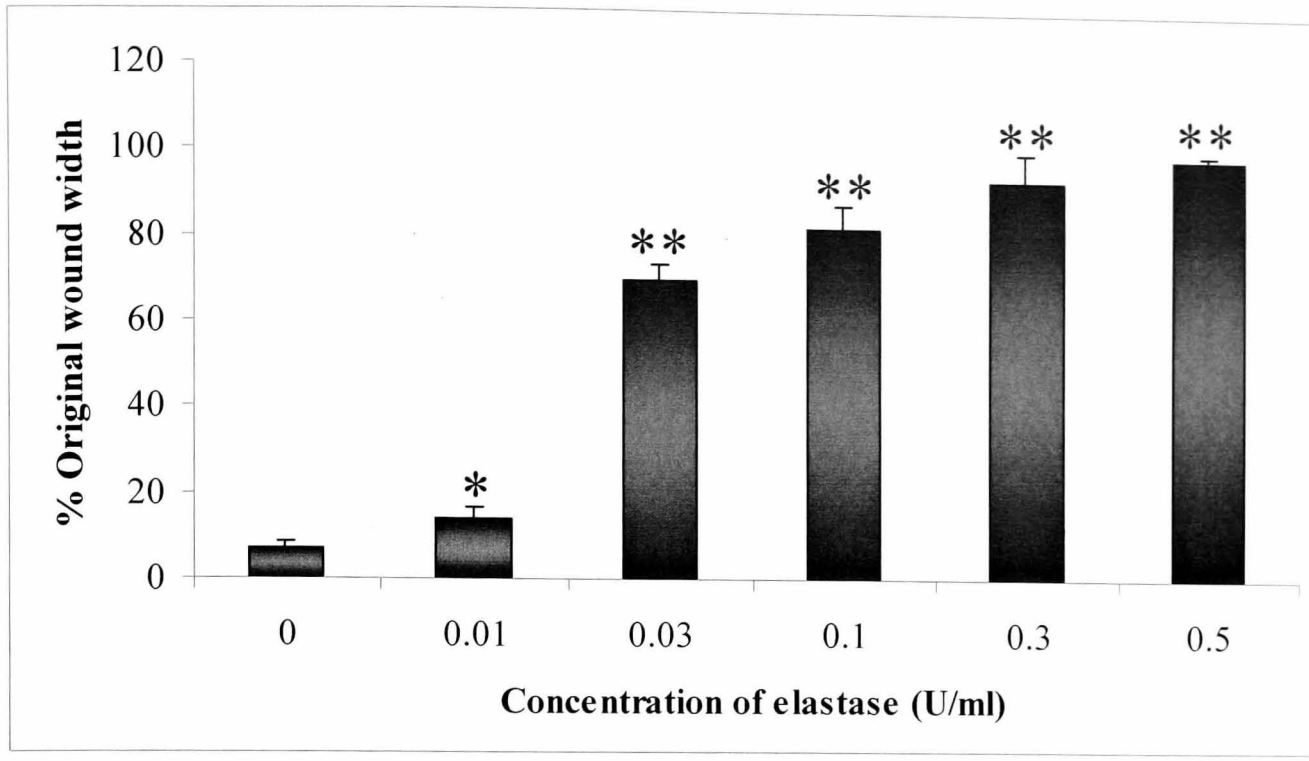

Figure 4.27. Effect of elastase on bronchial epithelial repair of 16HBE 14o$^-$ cells at 13 hours. Data represent mean ± SEM (*n=3*). * Indicates P<0.05 and ** indicates P<0.0001 compared to no elastase control.

The addition of elastase significantly inhibited wound repair in a dose-responsive manner: 0.01 U/ml elastase = 13.74 ± 3.13 %; 0.03 U/ml elastase = 69.70 ± 3.66 %; 0.1 U/ml elastase = 81.85 ± 5.35 %; 0.3 U/ml elastase = 93.36 ± 6.54 % and 0.5 U/ml elastase = 98.55 ± 0.89 %; compared to the untreated cell cultures, 6.75 ± 1.93 % at 13 hours, indicating almost complete wound repair.

4.4.11.1. Effect of elastase on LDH levels

In order to determine whether the effects of elastase on wound repair were due to cytotoxicity, an LDH assay was conducted on supernatants and cell lysates at 13 hours.

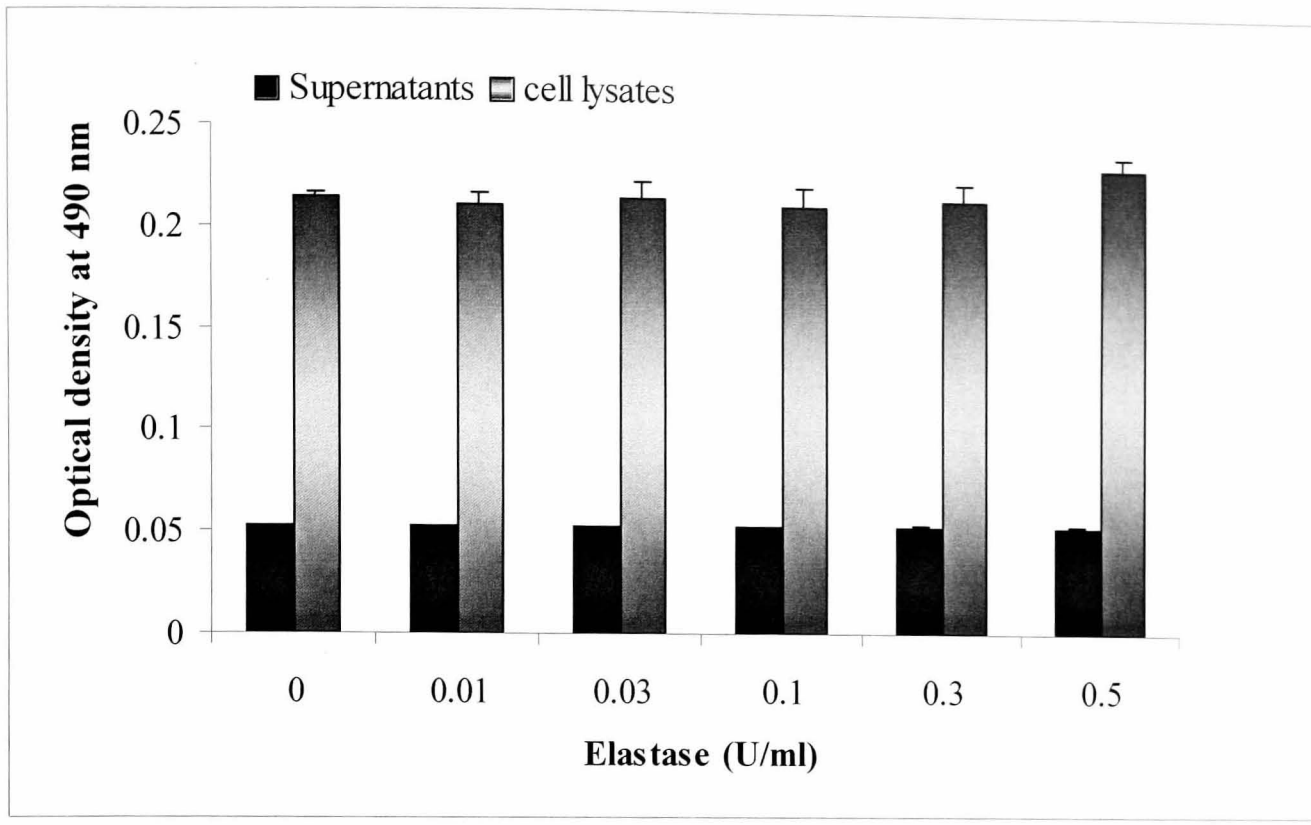

Figure 4.28. The effect of elastase on the levels of LDH in cell culture supernatants at 13 hours. Data represent mean ± SEM (*n=3*).

Elastase in the concentration range of 0-0.5 U/ml had no effect on the distribution of LDH between the supernatants and cell lysates indicating that the inhibition of wound repair was not due to cytotoxicity induced by this enzyme.

4.5. Summary of results

Neutralising antibodies to TF, fibrinogen and FXIII inhibited wound repair of both 16HBE 14o$^-$ and NHBE cells and corresponding non-immune control antibodies had no effect. Inhibition of wound repair of both 16HBE 14o$^-$ and NHBE cells corresponded to a significant increase in the levels of fibrinogen in cell culture supernatants and no effect on FXIII concentration. Inhibition of both FXa and thrombin significantly enhanced wound repair of 16HBE 14o$^-$ cells. Both exogenous and endogenous PGE_2 had no effect on wound repair of 16HBE 14o$^-$ cells. Finally, neutrophil elastase significantly inhibited wound repair of 16HBE 14o$^-$ cells.

4.6. Discussion

A recent report indicated that both migration and proliferation were involved in the repair of 16HBE 14o$^-$ cell monolayers (Zhu *et al.*, 2007). However, wound repair in the model described in this thesis was dependent on cell migration and not proliferation as there was no significant effect of MMC on wound repair, as previously reported for 16HBE 14o$^-$ cells (Howat *et al.*, 2002). The difference may relate to the different inhibitors of proliferation used. Zhu *et al.* used nocodazole compared to MMC in the current study. However, the stimulus for cell migration is not fully understood. It must be noted that as well as the use of MMC as a proliferation inhibitor, cell cycle kinetic markers including proliferating cell nuclear antigen (PCNA), Ki-67 (MIB-7) or bromodeoxyuridine (BRDU) may have been used as alternative and possibly more effective compounds.

The normal bronchial epithelium forms a highly regulated, virtually impermeable barrier through the formation of tight junctions at the apical perimeter of the cell composed of occludins and claudins, which prevent the passage of molecules and ions through the space between cells in order that materials must traverse cells by diffusion or active transport (Sparrow *et al.*, 1995). Structural integrity is maintained between cells by adherens junction proteins such as epithelial-cadherin (E-cadherin). Similarly, desmosomes are involved in the adhesion of adjacent cells and hemidesmosomes are responsible for the adhesion of epithelial cells to the basement membrane (Hackett *et al.*, 2007). Thus, it is likely that removal of epithelial cells as a result of mechanical wounding results in physical cleavage of adherens junctions and that the disruption of epithelial integrity may be the signal for cell migration. This concept is supported by the immunohistochemical images (*Chapter 3*) demonstrating the formation of lamellipodia at the leading edge of the cells indicating directional epithelial cell migration.

E-cadherin is recognised as one of the most important molecules in the maintenance of epithelial integrity and wound repair (Gumbiner, 1996; Takeichi, 1991). E-cadherin is a type I transmembrane glycoprotein of which the extracellular domain interacts homotypically with E-cadherin molecules on the surface of neighbouring cells to form calcium-dependent adherens junctions. The stabilisation of intercellular adhesion requires the cytoplasmic domain of E-cadherin, which associates with β-catenin, a dual

function protein which is linked to the cytoskeleton and plays a role in cell adhesion and gene expression (Maretzky *et al.*, 2005).

β-catenin activates the canonical Wnt pathway (Behrens *et al.*, 1996; Miller *et al.*. 1999). Following accumulation of β-catenin, it translocates into the nuclear compartment where it binds to T cell factor (Tcf) and lymphoid enhancer factor (Lef)-1 proteins and activates gene transcription (Miller *et al.*, 1999). Recent evidence indicates a role for β-catenin in wound repair of bronchial epithelial cells (Zhu *et al.*. 2007). It was demonstrated that glycogen synthase kinase 3β (GSK3β)/β-catenin signalling was involved in wound repair that was due to both proliferation and migration of 16HBE $14o^{-}$ cells. GSK3β is a multifunctional serine/threonine kinase that is known to phosphorylate and down-regulate β-catenin and is thereby a negative regulator of the Wnt signalling pathway (Doble *et al.*, 2003; Grimes *et al.*, 2001). Following inhibition of GSK3β activity, β-catenin accumulated and the rate of wound repair was enhanced. Moreover, mechanical wounding alone was demonstrated to cause inhibitory phosphorylation of GSK3β, which lead to accumulation of β-catenin and subsequent wound repair (Zhu *et al.*, 2007). Primary NHBE cells have been shown to express mRNA encoding Tcf and Lef-1 that are required for canonical Wnt cell signalling (Steel *et al.*, 2005). In the same study cell density was shown to influence β-catenin transcriptional activity, whereby a redistribution of activated β-catenin into the nuclear compartment was demonstrated in sub-confluent NHBE cells. The relevance of the effect of cell density is that when monolayers of epithelial cells are mechanically wounded, they may be considered sub-confluent.

Following neutralisation of E-cadherin, adherens junctions are weakened and cells become rounded and less adhesive in order to facilitate cell migration. It has been demonstrated that inhibition of E-cadherin function can be mediated through the shedding of its ectodomain as a result of cleavage by matrilysin (MMP-7) and stromelysin-1 (MMP-3) (Noe *et al.*, 2001). In alveolar epithelial cells, transfection with MMP-7 resulted in shedding of E-cadherin and accelerated cell migration suggesting that shedding of the E-cadherin ectodomain is required for epithelial wound repair. MMP-7 was reported to facilitate migration of surviving cells by promoting the re-organisation of cell-cell junctions (McGuire *et al.*, 2003). In bronchial epithelial cells,

expression of MMP-7 is upregulated following injury (Dunsmore *et al.*, 1998: Lopez-Boado *et al.*, 2001). Interestingly, TGF-β1 can upregulate the expression of MMPs (Lechapt-Zalcman *et al.*, 2006) and the total concentration of this growth factor was increased in response to wounding (*section 3.4.4.3*), possibly leading to shedding of E-cadherin. Moreover, trypsin is a product of injured epithelial cells and has been demonstrated to mediate the removal of the E-cadherin ectodomain and induce tyrosine phosphorylation of β-catenin (Takahashi *et al.*, 1997) and may therefore be involved in the initial stimulus for cell migration. Activation of *c-Met* by HGF has recently been associated with decreased E-cadherin-dependent cell-cell contacts (Reshetnikova *et al.*, 2007). Expression of *c-Met* was demonstrated in 16HBE 14o$^-$ cells (*section 3.4.4.4*) and was upregulated in response to wounding. Conversely, the same cell line was not shown to express HGF, however, HGF released from fibroblasts *in vivo* may bind to *c-Met* on epithelial cells to stimulate cell migration.

In asthma, the bronchial epithelium is highly fragile. Moreover, bronchial epithelial cells from asthmatic individuals appear to be more activated compared to non-asthmatics and the release of inflammatory mediators such as TNF-α is increased (Holgate *et al.*, 1999; Montefort *et al.*, 1993). TNF-α released from NHBE cells mediates cell-cell dissociation associated with disordered expression of E-cadherin and β-catenin (Tabibzadeh *et al.*, 1995). It has been suggested that modifications in E-cadherin binding to β-catenin may be involved in the weakening of the bronchial epithelium in asthma (Carayol *et al.*, 2002). Thus, in normal bronchial epithelial repair, removal of E-cadherin fragments and subsequent accumulation of β-catenin is beneficial in mediating wound repair, however, in asthma the continued release of pro-inflammatory mediators such as TNF-α and subsequent E-cadherin shedding may be detrimental.

It has been suggested that epithelial injury activates a repair response of basal cells targeted at the re-epithelialisation of the wound (Vignola *et al.*, 2000). Such a repair process requires the release of ECM components including fibronectin and collagens and of factors that establish a chemotactic gradient influencing the migration of bronchial epithelial cells. Another fundamental step in the repair process is the ability of cells to adhere to the ECM and then migrate towards the wound. This event relies

heavily upon integrins, which have the capacity to modulate the interactions between cell and ECM components (Sheppard, 2003).

Following injury *in vivo* and denudation of the basement membrane, a provisional matrix is formed which is composed of remnants of the original basement membrane such as laminin and collagen IV. Plasma exudate contains fibrin and fibronectin as well as other ECM proteins such as vitronectin. However, bronchial epithelial cells are capable of producing fibronectin and collagen IV and fibroblasts underlying the wound are important sources of proteoglycans, collagens, fibronectin and cytokines (McGowan, 1992; Shoji *et al.*, 1990). Besides being a major component of the provisional matrix and representing an important adhesive substrate for epithelial cells, fibronectin and its soluble fragments are active in promoting directional epithelial cell migration in the lung (Shoji *et al.*, 1989). Vitronectin, collagen and laminin are also described as potent stimuli for bronchial epithelial cell migration and have been demonstrated to promote directional migration of bovine tracheal epithelial cells *in vitro* (Rickard *et al.*, 1993). Epithelial cell migration over the provisional matrix is a complex process involving the adhesion and de-adhesion of the cells to matrix components. In order to migrate, epithelial cells must balance adhesive interactions with motility, which requires continual establishment and spreading of adhesive contacts.

Bronchial epithelial cells express a variety of integrin receptors including the collagen-laminin receptor ($\alpha 2$, $\alpha 3$ and $\alpha 6$ chains), fibronectin receptor (αv chain) vitronectin receptor (αv chain) and $\alpha 6\beta 4$ receptor (Montefort *et al.*, 1991; Rossi *et al.*, 1990). An increased expression of specific integrins including $\alpha v\beta 1$ and $\alpha v\beta 6$ occurs on the basal surface of regenerating epithelial cells after the deposition of fibronectin suggesting that fibronectin may induce integrin expression (Horiba *et al.*, 1994). Bronchial epithelial cells also express $\alpha 3\beta 1$ integrin, the counter-receptor of several ECM components including fibronectin, laminin and collagen. Since the cells in the vicinity of a wounded area produce ECM components and the deposition of ECM components on the basement membrane is followed by adhesion and spreading of bronchial epithelial cells eventually leading to wound repair, it has been suggested that the expression of $\alpha 3\beta 1$ integrin by these cells may play an important role in the regulation of their interaction with ECM components (Vignola *et al.*, 2000). Despite tight regulation of epithelial migration by integrins (Sheppard, 1996); these molecules themselves do not possess any

catalytic activity and are therefore unable to initiate signalling cascades independently. Instead, signal transduction from integrins to regulate migration occurs *via* several intermediate pathways (Parsons, 1996).

A variety of proteoglycans have been described on the surface of bronchial epithelial cells such as syndecan that can bind collagen and fibronectin and might therefore participate in adhesion and cell migration (Dufour *et al.*, 1986; Mette *et al.*, 1993). Moreover, the adhesion molecule CD44 can bind fibronectin and collagen and there is evidence to suggest that it might play a role in bronchial epithelial repair (Cichy *et al.*, 2002; Holgate, 2000).

Expression of the chemokine receptor CXCR3 has recently been demonstrated in NHBE and 16HBE 14o$^-$ cells and was shown to induce directional cell migration when activated by its ligand, I-TAC (Aksoy *et al.*, 2006). Thus, there are a number of possible mechanisms, which may provide the initial stimulus for epithelial cell migration and subsequent wound repair.

Restitution to an intact undifferentiated epithelial barrier following wounding can take as little as one hour in the guinea pig lung *in vivo* (Erjefalt *et al.*, 1997), whereas the time taken for wound repair in the human lung has not been determined. Under serum-free conditions, the rate of cell migration of controls as determined by the method described in *section 4.3.10* was 0.83 um/min and considerably greater than that reported in other studies whereby cell migration of A549 alveolar epithelial cells was 0.06 um/min (Galiacy *et al.*, 2003) and migration of primary bronchial epithelial cells cultured on collagen I-coated plastic ware was 0.34 um/min (Zahm *et al.*, 2000). Interestingly, Zahm et al. (2000) reported that the addition of HGF increased the rate of cell migration to 0.84 um/min, which is similar to that demonstrated in the current study in which cells were not exposed to exogenous motogenic factors and were not cultured on a collagen matrix.

Inhibition of wound repair in both 16HBE 14o$^-$ and NHBE cells by a blocking antibody to TF indicated that wound repair was dependent on TF expressed by these cells. However, the blocking of TF may not only inhibit initiation of the coagulation cascade

but also activation of PARs since TF-FVIIa-FXa can activate both PAR-1 and PAR-2 (Chambers *et al.*, 2002; Macfarlane *et al.*, 2001).

TF exists in microdomains on the cell surface with a mixed population of active and encrypted protein. Most cell surface TF is latent and simple exposure of the cell surface to circulating FVII is not sufficient to trigger coagulation. Latent TF describes the encrypted form of this coagulation factor, whereby the procoagulant activity of TF on the cell surface is suppressed (Bach, 2006). Hence the reason that under normal conditions, TF associated with the surface of blood cells does not trigger coagulation. Thus TF encryption represents a physiological mechanism for controlling the expression of cellular coagulant activity *in vivo*.

De-encryption of TF is associated with a release of calcium from internal stores, followed by a disruption in phosphatidylserine asymmetry. Phosphatidylserine has long been established to accelerate coagulation reactions on cell surfaces. It is believed that encrypted TF is sequestered in lipid rafts and the de-encrypted form is released into the liquid disordered phase of the membrane following cell stimulation (Bach, 2006).

Cell surface TF binds FVIIa to initiate coagulation or, alternatively, to trigger signalling through PARs. It has been suggested that TF:FVIIa mediated coagulation and cell signalling involve distinct cellular pools of TF, that signalling TF refers to the encrypted form and that de-encryption of TF is necessary to promote coagulation. Disulphide isomerisation has been demonstrated to facilitate a dynamic and reversible switch between these two distinct TF species (Ahamed *et al.*, 2006). The cell surface extracellular disulphide bond of TF is critical for coagulation and when targeted with protein disulphide isomerase (PDI), the bond is cleaved and coagulation is disabled. However, the disulphide bond was not found to be required for PAR-2 cleavage by TF:FVIIa. Ahamed *et al.* (2006) demonstrated that cleavage of the disulphide bond to inhibit coagulant function is also regulated by NO pathways and conclude that inhibition of TF:FVIIa signalling is feasible without impairing coagulation.

Neutralising antibodies to fibrinogen and FXIIIA significantly inhibited wound repair of both 16HBE 14o$^-$ and NHBE cells compared to untreated wounded cell culture controls and corresponding non-immune immunoglobulin controls. This signifies that these

coagulation factors are important in wound repair. Since the extrinsic coagulation cascade ultimately leads to fibrin formation and a fibrin matrix is required for wound repair (Erjefalt *et al.*, 1994), the fact that fibrinogen and FXIIIA play a functional role in 16HBE 14o$^-$ and NHBE wound repair suggests that wound repair is occurring *via* fibrin formation in this model and that a cross-linked fibrin matrix is essential for wound repair. In hindsight, it would have been useful to perform immunocytofluorescence for fibrin deposition in wounded monolayers of 16HBE 14o$^-$ cells in order to confirm the formation of fibrin in response to wounding.

Previous studies have reported that fibrinogen is synthesised and secreted by A549 alveolar epithelial cells and is demonstrated to significantly enhance wound repair (Haidaris, 1997). However, synthesis and secretion of this coagulation factor followed stimulation of these cells with the inflammatory mediators IL-6 and dexamethasone whereas wounding was the stimulus in the current model. There is a possibility that the fibrinogen released from bronchial epithelial cells in the current study may constitute part of the ECM and facilitate cell migration independently of conversion to fibrin (Guadiz *et al.*, 1997).

FXIII is a transglutaminase and exists in the blood as a heterotetramer (A_2B_2) of which FXIIIA contains the active site. FXIIIa catalyses the formation of isopeptide cross-links between glutamine and lysine side chains of adjacent polypeptides and its main function is to cross-link fibrin chains (Muszbek *et al.*, 1999). However, the enzyme also plays a role in the cross-linking of fibrin with other proteins including fibronectin, α_2-plasmin inhibitor and collagen leading to an increase in mechanical strength, elasticity and resistance to degradation of fibrin clots by plasmin and to the promotion of wound repair (Ichinose, 2001). There is accumulating evidence to support the concept that the interaction of cells with the ECM is essential for migration and subsequent wound repair. FXIII has been identified as a ligand for the integrins $\alpha9\beta1$ and $\alpha4\beta1$ which are transmembrane receptors that mediate cell-ECM interactions that are important in cell migration (Takahashi *et al.*, 2000) and of which $\alpha9\beta1$ is abundantly expressed by basal bronchial epithelial cells (Sheppard, 2003). The release of FXIII from bronchial epithelial cells and the anchoring of the fibrin clot to the ECM suggests that FXIII is directly involved in wound repair and this notion is confirmed by the fact that wound repair is inhibited in the absence of this enzyme.

In the current study, wound repair is dependent on cell migration, which is subsequently dependent on the formation of a cross-linked fibrin gel matrix. Blocking the formation of this matrix by antibodies to TF, fibrinogen and FXIII resulted in the accumulation of fibrinogen in cell culture supernatants, indicating not only that wounding was a strong stimulus for fibrinogen release but that when wound repair was inhibited, fibrinogen continued to be released but not consumed in the wound repair process. Despite inhibition of NHBE wound repair by anti-TF, there was no significant accumulation of fibrinogen released into cell culture supernatants. The fact that NHBE cells do not achieve full confluence in submerged cultures or posses the tight junctions of 16HBE $14o^-$ cells may provide an explanation for the differences in responses. Alternatively, it is possible that the fibrinogen released from NHBE cells was incorporated into the ECM independently of conversion to fibrin as for A549 alveolar epithelial cells (Haidaris, 1997). Analysis of the levels of D-dimers in cell culture supernatant levels would clarify whether or not fibrin was formed.

Conversely, inhibition of wound repair with the noted blocking antibodies did not induce any significant changes in the levels of FXIII in either 16HBE $14o^-$ or NHBE cell culture supernatants compared to the untreated wounded cell culture controls. Thus, FXIII is released in response to wounding but does not accumulate following the blocking of the coagulation pathway. The lower levels of FXIII may signify the binding of this protein to ECM components or that FXIII is less stable than fibrinogen and is released and subsequently degraded. An investigation of the interaction of FXIII with cultured fibroblasts demonstrated that cell associated activated FXIIIA is involved in the cross-linking of fibronectin and is subsequently internalised and degraded by fibroblasts (Barry *et al.*, 1990).

In hindsight, it must be noted that the experiments involving the effect of neutralising coagulation factor antibodies on the levels of fibrinogen and FXIII in supernatants derived from 16HBE $14o^-$ cells were flawed, as they did not include the corresponding isotype control antibodies.

Incubation of 16HBE $14o^-$ cells with FXa resulted in a concentration-dependent promotion of wound repair. Since FXa plays a central role in the coagulation cascade,

catalysing the conversion of prothrombin to thrombin which ultimately leads to the formation of fibrin (Davie *et al.*, 1991), it is conceivable that the addition of FXa enhanced the coagulation process on the cell surface.

FXa is a serine protease and is known to activate both PAR-1 and PAR-2 (Chambers *et al.*, 2002; Macfarlane *et al.*, 2001). Since activation of both PAR-1 and PAR-2 was shown to stimulate repair, it is possible that exogenous FXa enhanced repair by a PAR-mediated mechanism. Alternatively, incubation of HUVECS with FXa in a similar concentration range was previously shown to stimulate the release of NO as determined by the accumulation of cGMP in these cells which was inhibited by L-NAME, the NOS antagonist (Papapetropoulos *et al.*, 1998). The bronchial epithelium continuously produces NO, largely due to constitutive expression of iNOS (Xu *et al.*, 2006), and NO has been associated with cell migration and epithelial repair following injury. A recent study demonstrated that addition of an NO donor and stable transfection of NHBE cells with iNOS in order to mimic constitutive epithelial iNOS expression *in vivo* lead to a promotion of wound repair *via* activation of MMP-9 (Bove *et al.*, 2007). Thus, there is a possibility that wound repair occurred by FXa-mediated NO production.

Interestingly, incubation of 16HBE 14o$^-$ cells with two separate selective FXa inhibitors resulted in inhibition of wound repair only at low concentrations (0.05 μM) but enhanced wound repair at higher concentrations (0.1-1 μM). Given that FXa plays a crucial role in the coagulation cascade, it was therefore not expected that endogenous FXa would be found to inhibit repair. Since FXa binds to negatively charged phospholipids, two pools of FXa substrate exist, one of which is released from cells and is free in solution and the other which is membrane-bound (Papahadjopoulos *et al.*, 1964). It has been demonstrated that free FX determines the rate of FX activation and that free FX is the true substrate for TF-FVIIa in the extrinsic pathway of coagulation (Forman *et al.*, 1986). Thus, it is possible that the inhibition of wound repair at low concentrations was due to effects on FXa that was released into the cell culture supernatant and behaved in the same way as exogenously added FXa and that stimulation of wound repair was related to FXa that was phospholipid-bound.

In addition to its role in coagulation, FXa is also considered to mediate inflammatory events *via* PAR-1 and PAR-2. Activation of both PAR-1 and PAR-2 stimulates PGE_2

production by epithelial cells to initiate PGE_2-dependent bronchoprotection (Cocks *et al.*, 2000). However, in the current model, there is accumulating evidence to suggest that inhibition of wound repair by endogenous FXa is not mediated *via* PARs. Firstly, activation of PAR-1 and PAR-2 enhanced the rate of bronchial epithelial repair, as demonstrated in *section 5.4.1*; and data generated from immunoblotting in *Chapter 5* demonstrated that both PAR-1 and PAR-2 increased the release of fibrinogen, FXIII and D-dimers, at baseline and in response to wounding, indicating that PAR-stimulated wound repair involves the formation of fibrin. Secondly, PAR activation generates PGE_2 that has been demonstrated to enhance bronchial epithelial repair *in vitro* (Savla *et al.*, 2001). Thus, the observation that the FXa inhibitor enhances wound repair in the current model suggests that this effect is not *via* FXa-mediated PAR activation. In order to confirm this, it would have been useful to include an antagonist of PAR-1 and PAR-2 or alternatively, a neutralising PAR antibody.

The stimulatory effect on wound repair by the selective FXa inhibitor PD-031 was reversed by indomethacin in a concentration-dependent manner. Indomethacin inhibits COX activity and hence the conversion of arachidonic acid to PGE_2, the predominant prostanoid produced by bronchial epithelial cells (Savla *et al.*, 1997). Thus, when FXa was inhibited, PGE_2 (or other prostanoids) was apparently involved in the wound repair process as determined by inhibition of wound repair by indomethacin. Therefore, it is possible that endogenous FXa suppresses PGE_2 synthesis or that inhibition of FXa increases PGE_2 synthesis suggesting that PGE_2 may play a role in wound repair under these conditions.

In order for FXa activation, calcium is required to promote the binding of enzyme and substrate and subsequent catalysis requires a phospholipid membrane (Nesheim *et al.*, 1979; Rodgers *et al.*, 1983). Equally, PGE_2 production is dependent upon the presence of polyunsaturated fatty acids. Previous studies have demonstrated that tracheal epithelial cells are dependent on *serum-containing* medium for essential fatty acids to support PGE_2 synthesis. Cultured tracheal epithelial cells in serum-free medium were deficient in PGE_2 production at baseline and in response to stimuli, unlike cells cultured in medium supplemented with serum, which displayed greater content of arachidonic acid. Cells that were maintained in serum-free media contained a higher proportion of palmitoleic acid and oleic acid and decreased proportion of linoleic acid and arachidonic

acid indicating essential fatty acid deficiency. Both human and rabbit species of tracheal epithelial cells cultured in serum-free conditions demonstrated a loss of 85-90% of the arachidonic acid found in freshly isolated cells and cellular linoleic acid content was decreased by 80%. Culture medium supplemented with 5% FBS was shown to increase the linoleic acid and arachidonic acid content of cultured rabbit and human tracheal epithelial cells, but was not sufficient to restore the fatty acid composition of freshly isolated cells. Instead, full restoration was achieved by the addition of a commercial, non-serum source of lipids, excyte III, in combination with exogenous arachidonic acid which in turn lead to restoration of PGE_2 synthesis (Alpert *et al.*, 1991).

In this reductive model of 16HBE $14o^-$ and NHBE cells, all experiments were carried out in a serum-free environment in order to study the regulation of cellular functions in the absence of coagulation factors. The absence of serum has proven invaluable in uncovering a role for the contribution of bronchial epithelial cells alone to wound repair. However, in the case of experiments investigating a role for PGE_2, it appears that removal of serum from the medium is restricting the availability of membrane phospholipids containing arachidonic acid required for PGE_2 production. Moreover, the requirement of phospholipids for FXa activity suggests that FXa may be reducing the availability for arachidonic acid and thus inhibiting PGE_2 production even further.

Inhibition of prostanoid production by FXa has also been demonstrated in other cell types. FXa has been demonstrated to inhibit the synthesis of thromboxane A_2 (TXA_2), the predominant prostanoid of platelets (Sinha *et al.*, 1983) and is described as a potent inhibitor of prostacyclin (PGI_2) production in endothelial cells. In this cell type, PGI_2 is the predominant prostanoid produced (Sinha *et al.*, 1985). In both platelet and endothelial cells, the reaction catalysing the conversion of prothrombin to thrombin by FXa occurs on the cell surface and is dependent on the presence of calcium (Rodgers *et al.*, 1983; Sinha *et al.*, 1983).

The stimulatory effect of FXa on wound repair may be *via* coagulation and/or PAR activation but the inhibitory effect of endogenous membrane-bound FXa may be *via* competition for phospholipids, or alternatively *via* EPR-1, which has not yet been described on bronchial epithelial cells. EPR-1 was identified by Altieri *et al.* in 1995 and so far has only been localised to leukocytes and endothelial cells, where it emerged

as a regulator of FXa-mediated mitogenic signalling. Through the interaction with EPR-1, FXa has been demonstrated as a mediator of acute inflammation and subsequent tissue injury in a rat model *in vivo* (Cirino *et al.*, 1997). In order to determine whether FXa is mediated *via* EPR-1 in this model, it would first be necessary to perform immunohistochemical staining for this receptor in 16HBE 14o$^-$ and NHBE cells. In addition, EPR-1 peptide agonists and corresponding scrambled control peptides could be employed to investigate the effects of EPR-1 in 16HBE 14o$^-$ and NHBE wound repair. However, the existence of EPR-1 has been questioned since the initial identification of the gene. The current perception is that EPR-1 is either a unique property of specific cell lines or that the published EPR-1 cDNA contains sequence errors or includes sequences from clones that actually derived from *survivin* mRNA (Zaman *et al.*, 2000).

In order to further investigate the suppressant effect of FXa on PGE_2 production, it would be interesting to determine the levels of PGE_2 in cell culture supernatants following stimulation of 16HBE 14o$^-$ cells with the FXa inhibitor, PD-031 in the presence of indomethacin. Furthermore, the same experiment could be performed in the presence of serum or excyte III in combination with arachidonic acid. Indomethacin alone had no effect on wound repair confirming that PGE_2 is not produced under the current culture conditions. Thus, this experiment could be repeated using medium containing serum or excyte III to investigate the effects of endogenous PGE_2 on wound repair.

Savla *et al.* (2001) demonstrated that the addition of PGE_2 in the concentration range of 0-10 μg/ml stimulated wound repair of 16HBE 14o$^-$ cells, in a dose-dependent manner. However, the same concentration range of PGE_2 employed in this model had no effect on wound repair. Moreover, Savla *et al.* also reported that indomethacin inhibited wound repair in a dose-dependent manner indicating inhibition of endogenous PGE_2, whereas indomethacin had no effect on wound repair in the current model. It must be noted however, that wound repair in Savla's model was carried out in cell culture medium containing 10% FBS, increasing the availability of phospholipid for arachidonic acid release and subsequent PGE_2 production. Promotion of wound repair by PGE_2 was mediated *via* E-prostanoid (EP)-1 and EP-4 (Savla *et al.*, 2001), however, under serum-free conditions, mRNA for neither EP-1 nor EP-4 was detected by RT-

PCR (as discussed in *chapter 6*). It is therefore conceivable that these receptors are down regulated in the current model due to the lack of serum in the cell culture medium and provides an explanation for the fact that addition of exogenous PGE_2 had no effect on wound repair.

Interestingly, a thrombin inhibitor encoded UK-156,406 was also demonstrated to significantly enhance wound repair of 16HBE $14o^-$ cells. Given that thrombin is a vital component of the coagulation cascade by mediating the conversion of fibrinogen to fibrin and cleaving the FXIIIA subunit to initiate fibrin stabilisation by covalent cross-linking, it was not expected that endogenous thrombin would inhibit wound repair. Thrombin also has the capacity to activate PAR-1 -3 and -4, however there is accumulating evidence that the majority of its cellular effects occurs *via* PAR-1. Detrimental effects of PAR-1 activation by thrombin in vascular and extravascular compartments includes the excessive deposition of extracellular matrix proteins and ongoing coagulation activity leading to fibrosis (Chambers *et al.*, 2002). In view of this, the observation involving the thrombin inhibitor is therefore not likely to be mediated *via* PAR-1 activation by thrombin. Furthermore, PAR-1 activation in the current model lead to an increase in wound repair and an increase in release of coagulation factors and D-dimers (as described in *Chapter 5*), which implies that PAR-stimulated wound repair involved fibrin formation, supporting the concept that the effect of the thrombin inhibitor is not PAR-mediated. Since thrombin activation also requires a phospholipid cell surface, it is likely that endogenous thrombin may also be having a suppressant effect on PGE_2 production for the same reasons as explained for FXa. Thus, by inhibiting thrombin, this may increase the availability of phospholipids in the membrane for production of PGE_2 and subsequent wound repair. It would therefore be interesting to measure the levels of PGE_2 in cell culture supernatants of cells that were stimulated with UK-156,406 to confirm this effect.

Elastase significantly inhibited repair of bronchial epithelial cell layers at the same concentrations that were shown in *Section 3.4.7* to deplete the supernatant of fibrinogen and FXIII. Proteolytic degradation of these essential coagulation factors may therefore limit the repair response. Alternatively, elastase has been shown to disarm PAR-1 (Renesto *et al.*, 1997) and PAR-2 (Chignard *et al.*, 2006). Studies have investigated the effects of neutrophil elastase (NE), cathepsin G (CG) and elastase from *Pseudomonas*

aeruginosa (Epa) in 16HBE 14o$^-$ cells, A549 alveolar epithelial cells and a constitutive human PAR-2 expressing cell line (KNRK/PAR-2). None of the noted proteases activated any of these cell lines as determined by cytosolic calcium measurements, however following exposure of cells to these proteases, cells were subsequently unresponsive to trypsin. It must be noted that the concentration of elastase that has previously been demonstrated to cleave PAR-2 (500 nM) (Dulon *et al.*, 2003) is 10-fold higher than that employed in the current study (50 nM) whereas similar concentrations of elastase have previously been shown to cleave FX (23 nM) (Turkington, 1991) and FVII (18 nM) (Anderssen *et al.*, 1993). A cytotoxic effect of elastase was ruled out as determined by LDH release assays. This observation is consistent with previous reports that epithelial cells are resistant to cytolytic injury by elastase (Chung *et al.*, 1991; Peterson *et al.*, 1995). Therefore, proteolysis of essential coagulation factors seems the most likely explanation for the observed inhibition of repair in the presence of elastase. In order to confirm this and to rule out an effect on PARs, it would be necessary to pre-incubate bronchial epithelial cells with neutrophil elastase prior to challenge with PAR peptides and to monitor the wound repair response.

The significant reduction by elastase of the levels of coagulation factors in cell culture supernatants and subsequent concentration-dependent inhibition of wound repair supports the notion that coagulation factors are essential to wound repair, which is in accordance with the observations investigating the effect of blocking antibodies to the coagulation factors.

The major source of elastase in the infected, inflamed human lung is the neutrophil (Cohen *et al.*, 1983). Neutrophils migrate from the blood vessel into the airway lumen during infection and inflammation and in doing so, they traverse both the endothelium and the epithelium (Hiemstra *et al.*, 1998). Neutrophils have previously been shown to damage the bronchial epithelium *in vitro* and *in vivo*. Activated neutrophils can directly contribute to increased epithelial permeability as determined by transepithelial electrical resistance measurements (Milks *et al.*, 1986; Peterson *et al.*, 1995). It has been demonstrated that neutrophils alter epithelial barrier integrity (Nash *et al.*, 1987; Sugahara *et al.*, 1986) and gap formation in epithelial monolayers induced by neutrophil elastase has been reported (Kercsmar *et al.*, 1993). Neutrophil elastase is reported to cause epithelial detachment from the ECM (Amitani *et al.*, 1991; Harlan *et al.*, 1981;

Van Wetering *et al.*, 1997), however, there are several studies that did not demonstrate any significant cell detachment despite elastase-induced epithelial permeability (Peterson *et al.*, 1995; Rickard *et al.*, 1992).

Neutrophil elastase also has the potential to further alter epithelial integrity indirectly by inducing the expression of the neutrophil chemoattractant IL-8 in bronchial epithelial cells (Nakamura *et al.*, 1992) and it is now recognised that IL-8 regulation by neutrophil elastase occurs in part through the cell surface membrane bound toll-like receptor 4 (Devaney *et al.*, 2003). Since IL-8 is a potent chemoattractant for neutrophils (Kunkel *et al.*, 1991), elastase contributes to sustained epithelial damage. Thus elastase both damages the epithelium and inhibits its repair. This may be relevant in severe asthma associated with viral infection where neutrophils dominate the inflammatory response (Fahy *et al.*, 1995; Holgate *et al.*, 2006; Vignola *et al.*, 1998; Wenzel *et al.*, 1999; Wenzel *et al.*, 1997).

Chapter 5.

Effect of PARs on Wound Repair & Coagulation Factor Expression

5. Effect of protease activated receptors (PARs) on wound repair and coagulation factor expression

5.1. Introduction

5.1.1. PARs

PARs are a group of G-protein-coupled, seven transmembrane domain, cell surface receptors that enable cells to monitor extracellular proteolytic activity. To date, four PARs, namely PAR-1, PAR-2, PAR-3 and PAR-4, have been identified and cloned and are widely expressed on cells in blood vessels, connective tissue, leukocytes, epithelium and many cell types within the airway (Berger *et al.*, 2001). PAR-1 and PAR-2 are abundant in the bronchial epithelium and have been implicated in asthma (Cocks *et al.*, 2001; Knight *et al.*, 2001).

Unlike conventional agonist-receptor systems, PAR activation is dependent on the specific cleavage of the amino-terminus of the receptor. The newly formed amino terminus then functions as a 'tethered ligand', docking and interacting with the second extracellular loop of the PAR to trigger the sequence of events involving G-protein dissociation that confers receptor activation (*figure 5.1*) (Macfarlane *et al.*, 2001; O'Brien *et al.*, 2001).

The predominant endogenous activators of PARs are thrombin (activating PAR-1, PAR-3 and PAR-4), trypsin and tryptase (activating PAR-2 and PAR-4) (Bohm *et al.*, 1996; Dery *et al.*, 1998; Ishihara *et al.*, 1997; Nystedt *et al.*, 1994). PAR-2 is also activated by coagulation factors VIIa and Xa, but not thrombin (Camerer *et al.*, 2000). The transient TF:FVIIa:FXa complex also activates PAR-1 and PAR-2; signifying that the TF:FVIIa initiated coagulation cascade is inseparably linked to PAR activation and cell signalling (Chambers *et al.*, 2002). It is now well established that short, synthetic peptide analogues that mimic the amino acid sequence of the PAR-tethered ligand can also activate PAR-1 (encoded SFLLRN), PAR-2 (encoded SLIGKV) and PAR-4 (encoded GYPGQV), but interestingly, not PAR-3 (*figure 5.1*) (Macfarlane *et al.*, 2001). These synthetic agonists display a greater selectivity for their receptor than do endogenous

ligands such as trypsin and thrombin and they have proven to be an invaluable tool in establishing a role for the PARs *in vivo*.

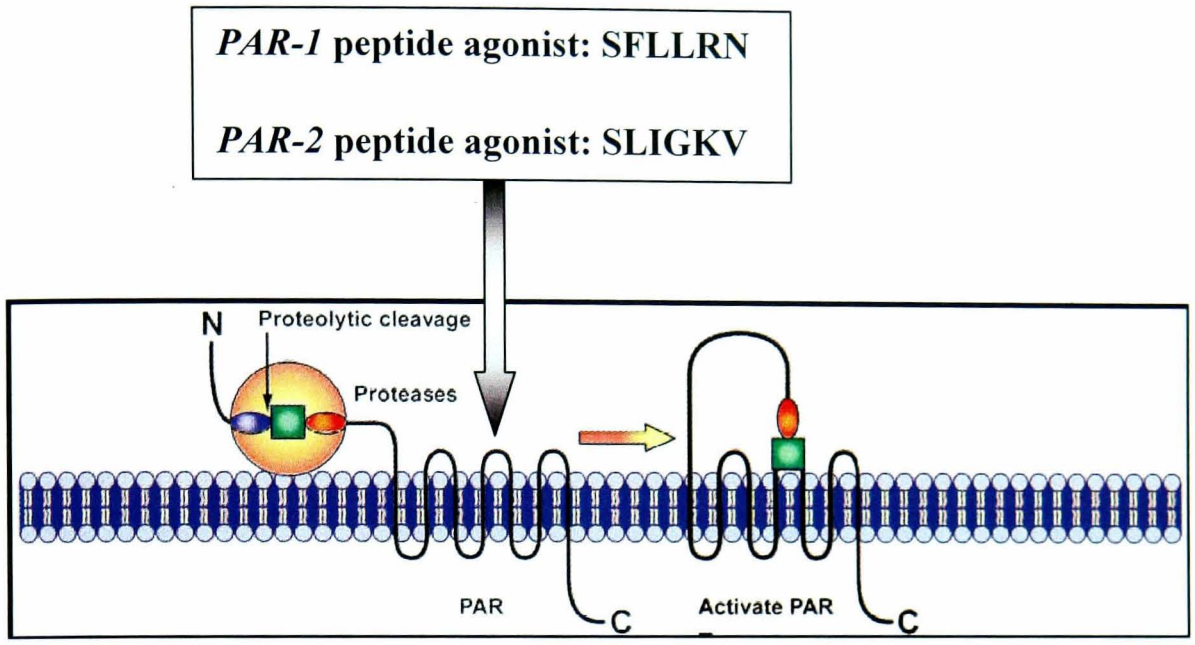

Figure 5.1. PAR activation by synthetic peptide agonists.
Peptide agonists, consisting of a short sequence of amino acids encoding the PAR-tethered ligand bind directly to the second extracellular loop of the PAR receptor, resulting in its activation.
Adapted from the review by Reed *et al.* (2004).

Conversely, some proteases have the potential to *inactivate* PARs by cleaving the carboxyl terminus and removing the tethered ligand from the receptor. In doing so, activation of the receptor by PAR activators is prevented. Neutrophil elastase can inactivate PAR-1, PAR-2 and PAR-3 (Chignard *et al.*, 2006; Parry *et al.*, 1996). Inactivation of PARs involves desensitisation of the receptor and is reported to be independent of receptor phosphorylation and receptor internalisation (Lan *et al.*, 2002). Elastase-mediated PAR inactivation may be biologically relevant in airway inflammatory diseases such as severe asthma, cystic fibrosis, acute lung injury (ALI) and ARDS where the levels of this pro-inflammatory enzyme are reported to be elevated in the extracellular spaces (Cochrane *et al.*, 1983; Dulon *et al.*, 2005; Holgate *et al.*, 2006; Lee *et al.*, 2001). Moreover, neutrophil elastase has been demonstrated to induce apoptosis of three different lung epithelial cell types BEAS-2B, human small airway epithelial (HSAE) cells and primary human alveolar type II (HAEC) cells through PAR-1 dependent modulation of intrinsic apoptotic pathway *via* alterations in mitochondrial permeability and by modulation of *c*-Jun kinase (JNK) and Akt, a serine/threonine kinase important in cell survival (Ginzberg *et al.*, 2004; Suzuki *et al.*, 2005).

The disarming cleavage sites on PAR-2 by various proteases including neutrophil elastase, cathepsin G and proteinase 3 have been determined by mass spectrometry and are indicated in *figure 5.2* (Chignard *et al.*, 2006).

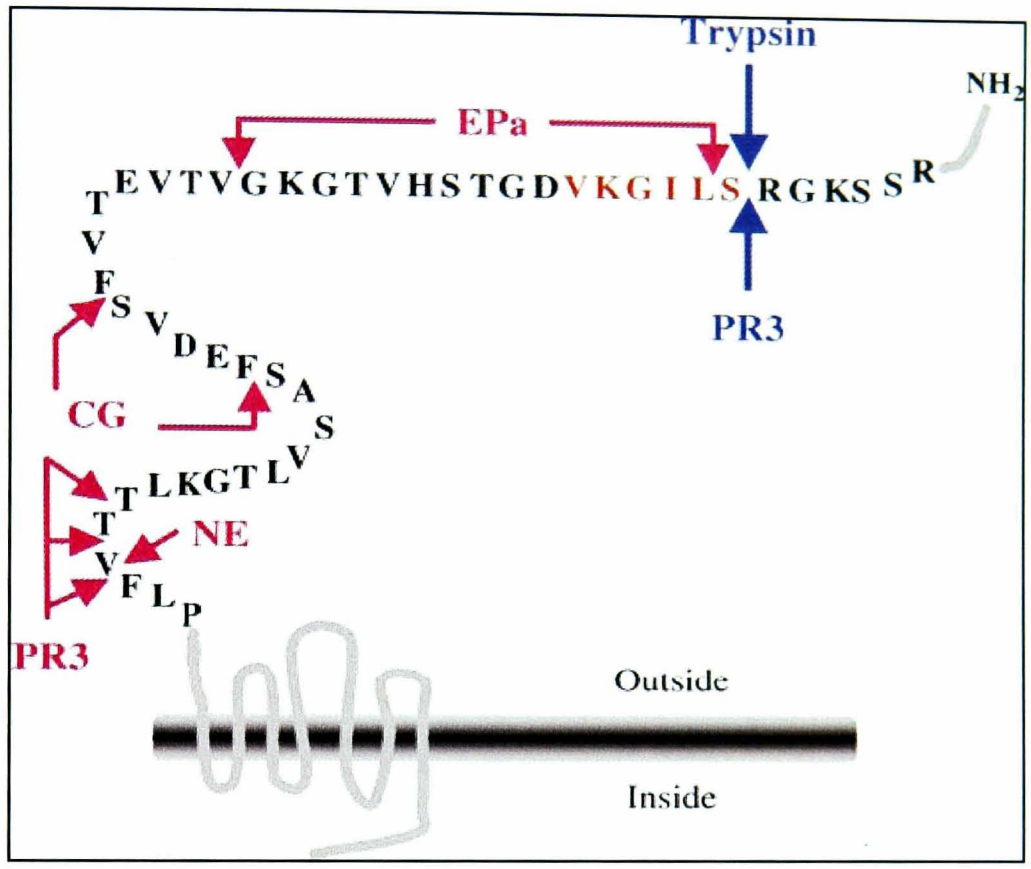

Figure 5.2. Representation of the N-terminal exodomain of PAR-2 expressed by epithelial cells showing the potential cleavage site of serine proteases. Activation and disarmament by proteases are indicated in blue and pink respectively. The amino acid sequence shown in red corresponds to the tethered activating sequence, which is revealed following activation cleavage of PAR-2 at Arg^{36}-Ser^{37}. Epa, elastase from Pseudomonas aeruginosa; CG, cathepsin G; NE, neutrophil elastase; PR3, proteinase 3 (Chignard et al., 2006).

5.1.1.1. Activation of PARs by exogenous proteases

Airborne allergens, including house dust mite faecal pellets, fungi, pollen and occupational dust have been shown to stimulate the release of proinflammatory cytokines *in vitro* (Hassim *et al.*, 1998; King *et al.*, 1998; Wan *et al.*, 1999) and can induce hyper-reactivity of the airways (*figure 5.3*) (Gavett *et al.*, 2001; Riccioni *et al.*, 2001). There is now evidence to suggest that these inhaled proteases may stimulate such responses such as the release of inflammatory mediators through the activation of the PARs. For example, house dust mite serine protease allergens from Der P3 and Der P9 have been shown to interact with PAR-2 (Asokananthan *et al.*, 2002; Sun *et al.*, 2001).

A wide range of proteases is known to be present in the lungs of atopic individuals suggesting that PARs are likely to be important in respiratory diseases such as asthma.

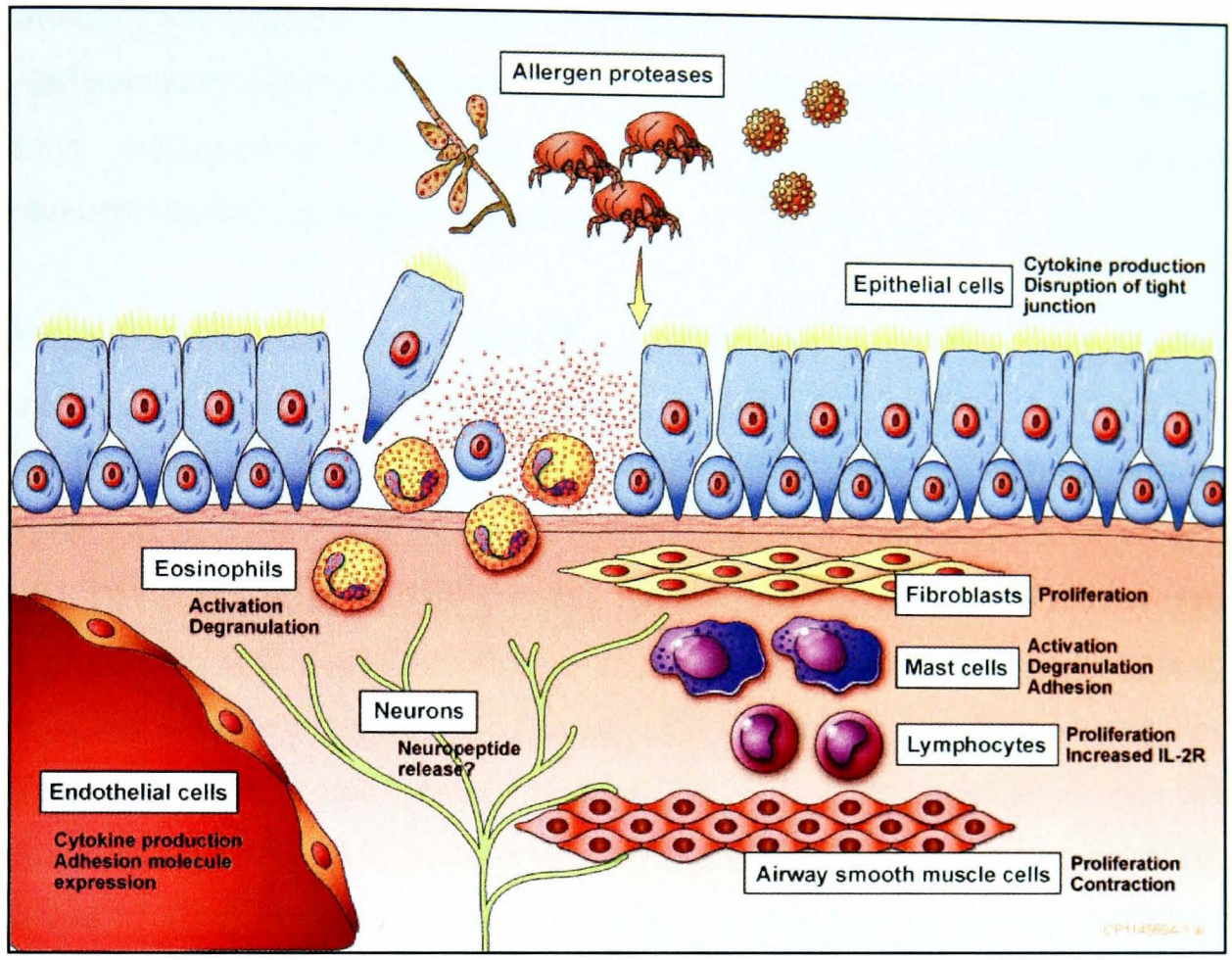

Figure 5.3. PAR-mediated inflammation of the airways: cellular responses.
Allergen proteases such as the house dust mite activate the PAR receptors, disrupt epithelial tight junctions, recruit inflammatory cells such as eosinophils into the airways and induce expression of proinflammatory cytokines. This is typical of the events that occur in the asthmatic airways. The normal bronchial epithelium is also exposed to allergen proteases, however, inflammation is overcome by mechanisms of normal bronchial epithelial repair (Reed *et al.*, 2004).

5.1.1.2. Pro-inflammatory response of PAR activation

PARs are present on almost all of the cells involved in asthma, including mast cells, eosinophils, neutrophils, monocytes, macrophages, lymphocytes, smooth muscle, endothelium, fibroblasts, epithelial cells and neurons (Reed *et al.*, 2004). Asthmatic subjects have been reported to express an increase in PAR-2 on bronchial epithelial cells compared to non-asthmatics (Knight *et al.*, 2001) but not on smooth muscle or alveolar macrophages (Roche *et al.*, 2003). Activated PARs couple to G-signalling cascades that increase phospholipase C levels, which in turn leads to an increase in intracellular calcium levels (Berger *et al.*, 2001; Camerer *et al.*, 2002; Schechter *et al.*,

1998). The effects of increased intracellular calcium levels lead to events such as the secretion of inflammatory mediators from epithelial cells, degranulation of mast cells and airway smooth muscle contraction. Gene transcription produces integrins, chemokines and cytokines, as well as COX-2. Activation of PARs *in vivo* mediates the pro-inflammatory effects associated with chronic inflammation in asthma, including oedema, angiogenesis, fibrosis, enhanced IgE production, leukocyte infiltration, cytokine production and hyperresponsiveness.

The bronchial epithelium expresses PAR-1, PAR-2 and PAR-4. Activation of PAR-2 on epithelial cells is associated with the release of IL-6, IL-8, MMP-9 and PDGF, all of which have the potential to induce or prolong inflammation (Shimizu *et al.*, 2000; Vliagoftis *et al.*, 2000). Expression of PAR-1 and PAR-2 has been demonstrated in 16HBE 14o$^-$ cells and activation of PAR-2 but not PAR-1 was demonstrated to increase IL-8 production in this cell line (Page *et al.*, 2003). Moreover, all four members of the PAR family have been demonstrated in the A549 and BEAS-2B epithelial cell lines, as well as in primary human bronchial epithelial cells (Asokananthan *et al.*, 2002). Activation of PAR-1, PAR-2 and PAR-4 by synthetic peptide agonists stimulated the secretion of IL-6, IL-8 and PGE_2 from A549, BEAS-2B and primary NHBE cells. These processes were accompanied by changes in intracellular calcium ions concentration. In addition PAR-2 activation resulted in the secretion of GM-CSF. In support of this, it has been demonstrated that mice deficient in PAR-2 display delayed inflammatory responses to tissue trauma (Lindner *et al.*, 2000). Furthermore, polymorphisms in PAR-2 alter sensitivity to agonist activation and can modulate inflammatory responses (Compton *et al.*, 2000). The fact that asthma is associated with increased expression of PAR-2 suggests that PAR-2 might be involved in airway inflammation. However, it is not known whether increased expression reflects a contribution to asthma pathology or an attempt by the epithelium to modify the inflammatory response.

5.1.1.3. A protective role for PARs

It is well established that PAR-2 is involved in cytoprotection in the pancreas and upper small intestine. PAR-2 is abundantly expressed on epithelia in the pancreatic duct (Nguyen *et al.*, 1999) and intestine (Kong *et al.*, 1997) and its activation leads to a

variety of cytoprotective effects, largely mediated by the release of PGE_2 from the epithelia (Cocks *et al.*, 2000). However, Cocks *et al.* have revealed that a similar PAR-2 dependent cytoprotective system also operates in the epithelia of the airways, whereby PAR-2 is involved in the growth of the normal epithelium and the restitution of the damaged epithelium following injury (Cocks *et al.*, 1999; Cocks *et al.*, 2000). Firstly, using an antibody directed against the carboxy terminus of mouse PAR-2 it was demonstrated by confocal fluorescence microscopy that specific PAR-2 immunostaining was localised to epithelial cells, often within the cytoplasm and to smooth muscle and fibroblasts in the submucosa of the mouse bronchus. Administration of a PAR-2 peptide agonist, mimicking the tethered ligand, resulted in concentration-dependent, rapid-onset and near-maximal relaxation of mouse bronchial rings, which had been pre-contracted by carbachol, the muscarinic agonist. These relaxations were abolished by removal of the epithelium or by inhibition of COX. The effects of relaxation due to nitric oxide were ruled out since relaxation was unaffected by the nitric oxide synthase inhibitor, L-NAME. Furthermore, addition of PGE_2, the most predominant prostanoid released from the bronchial epithelium, was found to exert powerful relaxation in mouse bronchi. Similarly, Pavord *et al.* (1995) demonstrated that endogenous PGE_2 conferred bronchoprotection against spasmogenic agents (Pavord *et al.*, 1995); and that inhaled PGE_2 inhibited both the early and late response to allergen-induced bronchial reactivity (Pavord *et al.*, 1993). A cytoprotective role for PAR-1 mediated by PGE_2 has been demonstrated in gastric epithelial cells (Toyoda *et al.*, 2003), however, there is little evidence as yet of PAR-1 mediated cytoprotection in the bronchial epithelium.

Since enzymic activation of PARs is irreversible, rapid resensitisation is essential for maintaining tissue-responsiveness to PAR-activating proteases. Turnover of cloned PAR-2 expressed in selective cell lines is rapid and depends on the synthesis of new protein in addition to trafficking of pre-formed receptors from intracellular pools (Bohm *et al.*, 1996; Dery *et al.*, 1998). Cocks *et al.* (1999) showed that PAR-2 mediated relaxations in mouse bronchi returned rapidly, 45 minutes after desensitisation to trypsin. These findings, in addition to the fact that PAR-2 is often localised to the cytoplasmic regions of epithelial cells suggest that there is rapid turnover of PAR-2 from intracellular stores in the airway epithelium. Moreover, the capacity of epithelial cells to rapidly recover their sensitivity to PAR-2 agonists following receptor

desensitisation indicates that epithelial PAR-2 participates in bronchoprotection. PAR-2 mediated relaxation was also observed in the airways of other species, including the rat and guinea pig (Cocks *et al.*, 1999).

Since PAR-2 is also expressed in the subepithelium, particularly on smooth muscle cells (D'Andrea *et al.*, 1998), the dual compartment model may explain the complex role of PAR-2 in the airways. It has been argued that epithelial and subepithelial PAR-2 could be differentially regulated by specific tryptic enzymes released in each compartment, i.e., epithelial trypsin and subepithelial mast-cell tryptase. Cocks *et al* (1999) demonstrated colocalised immunohistochemical staining of trypsin with PAR-2 in human bronchial epithelium, providing evidence that trypsin is the endogenous activator of epithelial PAR-2. They predict that the protective effects of epithelial PAR-2 normally overrides any proinflammatory effects of subepithelial PAR-2 activation, and that disruption of the epithelial barrier compromises the normal balance between the two compartments (Cocks *et al.*, 1999).

In this chapter, the effects of PAR-1 and PAR-2 agonists on wound repair, coagulation factor release and fibrin formation were investigated.

5.2. Aims and objectives

The initial aim was to investigate the effect of PAR-1 and PAR-2 on wound repair of 16HBE 14o$^-$ cells by use of kinetic video microscope analysis following stimulation of cells with synthetic PAR peptide agonists. Subsequent aims were to determine the effect of PAR-1 and PAR-2 activation on the release of coagulation factors into supernatants derived from 16HBE 14o$^-$ cells of unwounded and wounded cell monolayers. Moreover, it was imperative to identify a role for PAR-1 and PAR-2 in fibrin formation by analysis of D-dimers in 16HBE 14o$^-$ cell culture supernatants from unwounded and wounded monolayers. Finally, it was of interest to investigate the effect of PAR activation in the presence of the COX inhibitor indomethacin on coagulation factor release and fibrin formation in order to determine whether these effects were mediated *via* PGE_2.

5.3. Methods

5.3.1. Stimulation of 16HBE 14o$^-$ cells with PAR peptide agonists and immunoblotting

16HBE 14o$^-$ cells were cultured for 48 hours on 24-well plates until fully confluent and quiesced for 16 hours in serum-free MEM-ITS. Prior to experiments, MEM-ITS was refreshed. Synthetic PAR-1 peptide agonist (TFRIFD-amide, 10 mM stock dissolved in DMSO), PAR-2 peptide agonist (SLIGKVD-amide, 10 mM stock dissolved in DMSO) and corresponding scrambled PAR-1 control peptide (FTRIFD-amide, 10 mM stock dissolved in DMSO) and scrambled PAR-2 control peptide (LSIGKVD-amide, 10 mM stock dissolved in DMSO) and indomethacin were added to cells in a final concentration of 400 uM (PAR peptides) and 10 μM (indomethacin), in a volume of 250 μl per well and the plate was incubated for 30 minutes at 37°C. For cells that were exposed to a PAR peptide agonist in the presence of indomethacin, cells were pre-treated with indomethacin for 30 minutes at 37°C prior to the addition of PAR peptide agonist. Peptides were synthesised by Peptide Protein Research Ltd (Hampshire, UK). A final concentration of 0.04% DMSO was also included as a control. Cells were mechanically wounded with a P2 Gilson pipette tip in a cross-hatch manner with different degrees of wounding as described in *section 2.2.5* and returned to the incubator at 37°C for 2 hours. Supernatants were removed from cells and centrifuged for 7 minutes at 670 x *g* (ALC PK120 Centrifuge, Winchester Virginia, USA) to remove cell debris. Cell culture supernatants were then analysed by immunoblot for fibrinogen, FXIIIA and D-dimers as described in *section 3.3.3*.

5.3.2. Kinetic analysis of wound repair of 16HBE 14o$^-$ cells

Following stimulation of 16HBE 14o$^-$ cells with PAR peptide agonists as described in *section 5.3.1*, cells were mechanically wounded with a P2 Gilson pipette tip, one horizontal scrape per well. Immediately after wounding, the plate was placed on the heated stage at 37°C of the inverted wide field kinetic video microscope (Zeiss Axiovert 200 m; Carl Zeiss Ltd, Hertfordshire, UK) and Volocity® software was used to take images of the same section of wound for 13 hours, with time intervals of 15 minutes.

Volocity® software was also used to analyse wound repair by taking 10 measurements across the wound at each time point up to 13 hours. (For movie clip of wound repair, refer to CD attached to back cover of thesis).

5.4. Results

5.4.1. Effect of PARs on wound repair

The role of PARs in bronchial epithelial repair is incompletely understood, therefore it was of interest to investigate the direct effect of PARs in wound repair. In order to activate the PARs, short synthetic peptide agonists that mimic the 'tethered' region of the receptor were used. Wound repair was monitored in 'real-time' by kinetic video microscopy. The advantage of this technique is that it allows the migratory rate of epithelial cells to be calculated.

5.4.1.1. PAR-1

In order to activate PAR-1, the synthetic PAR-1 peptide agonist, TFRIFD-amide was added. A corresponding PAR-1 control peptide agonist, FTRIFD-amide with the first two amino acids in reverse order was included to confirm the selectivity of the PAR-1 peptide agonist for its receptor. A final concentration of 400 μM was used, as the potency of the peptides is very low and 400 μM was previously used to induce the release of cytokines in primary and human bronchial epithelial cell lines (Asokananthan *et al.*, 2002).

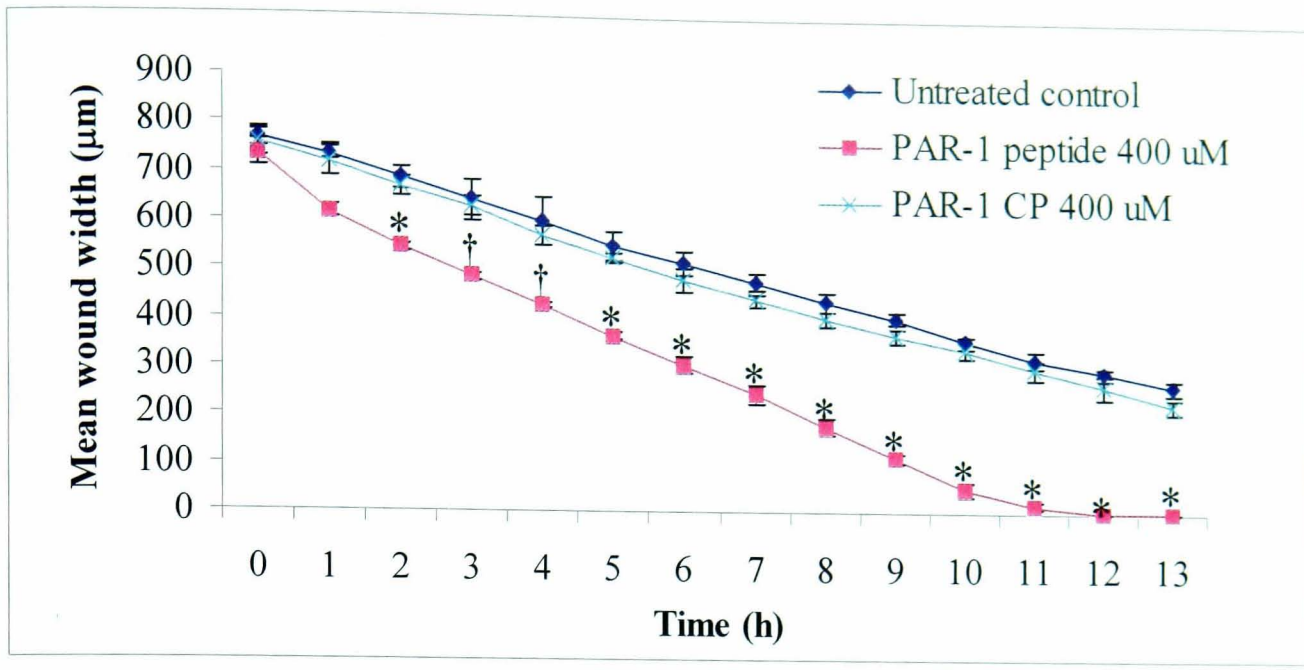

Figure 5.4. Effect of a PAR-1 peptide agonist on the rate of wound repair of 16HBE 14o$^-$ cell layers. PAR-1 CP denotes PAR-1 control peptide agonist. Data represent mean ± SEM (*n=3*). † Indicates P<0.05 and * indicates P<0.005 compared to no drug control.

With respect to the untreated cell cultures, the mean wound width at 13 hours was 263.58 ± 14.82 µm compared to 768.28 ± 19.89 µm at 0 hours, indicating 34.31% of the original wound width remained open. The PAR-1 control peptide (400 µM) had no effect on wound repair compared to the no drug control, which confirmed that it did not activate the PAR-1 receptor. The addition of PAR-1 peptide agonist (400 µM) significantly enhanced wound repair and at 12 hours, wound repair was complete.

5.4.1.2. PAR-2

In order to activate PAR-2, the synthetic PAR-2 peptide agonist, SLIGKVD-amide was added. A corresponding PAR-2 control peptide agonist, LSIGKVD-amide with the first two amino acids in reverse order was included to check the selectivity of the PAR-2 peptide agonist for its receptor. A concentration of 400 µM was added, as the potency of the peptides is very low and 400 µM was previously used to induce the release of cytokines in primary and human bronchial epithelial cell lines (Asokananthan *et al.*, 2002).

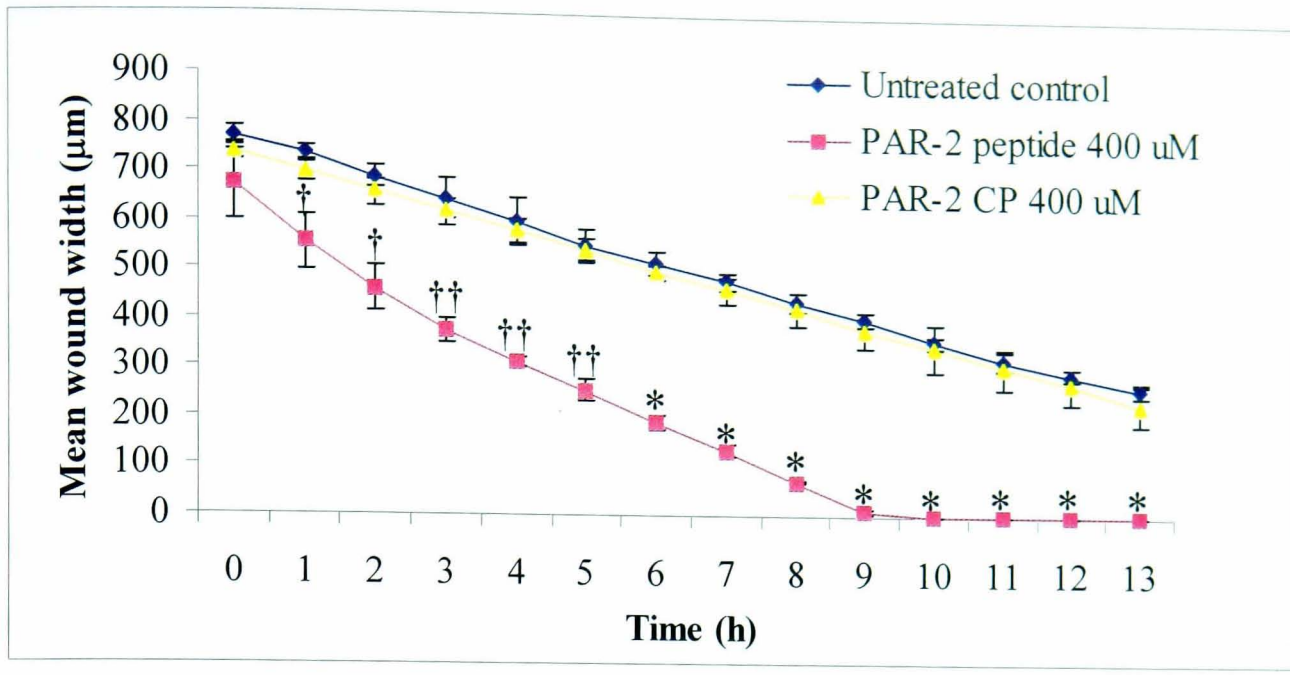

Figure 5.5. Effect of a PAR-2 peptide agonist on the rate of wound repair of 16HBE 14o$^-$ cell layers. PAR-2 CP denotes PAR-2 control peptide. Data represent mean ± SEM (*n=3*). † Indicates P<0.05, †† indicates P<0.005 and * indicates P<0.0005 compared to no drug control.

With respect to the untreated cell cultures, the mean wound width at 13 hours was 263.58 ± 14.82 µm compared to 768.28 ± 19.89 µm at 0 hours, indicating 34.31% original wound width remained open. The PAR-2 control peptide (400 µM) had no effect on wound repair compared to the no drug control, which confirmed that it did not activate the PAR-2 receptor. The addition of PAR-2 peptide agonist (400 µM) significantly enhanced wound repair and at 11 hours, wound repair was complete.

In terms of wound repair, the effect of PAR-2 was significantly greater than that of PAR-1 (P<0.05) between the time points of 3 hours and 10 hours.

The average migratory rate of 16HBE 14o$^-$ wound repair was 0.83 µm/min in control wells using the culture methods as described in *section 4.3.10*. However, when wound repair was monitored on the heated stage of the kinetic video microscope as described in *section 5.3.2*, the migratory rate of wound repair was 0.65 µm/min signifying differences in culture conditions. Epithelial cell migration *in vivo* is reported to be rapid (2-3 µm/min) following mechanical deepithelialisation (Erjefalt *et al.*, 1995). Understandably the migration rate is slower *in vitro*, however, the values are somewhat impressive considering the reductive nature of the current model.

5.4.2. Concentration of fibrinogen in cell culture supernatants

5.4.2.1. Effect of PAR-1

Since the activation of PAR-1 by a PAR-1 peptide agonist enhanced wound repair (*section 5.4.1.1*), it was of particular interest to investigate the effects of PAR-1 activation on the release of coagulation factors into cell culture supernatants. If wound repair was occurring by fibrin formation as a result of PAR-1 activation, it was expected that the concentration of coagulation factors and D-dimer fragments in cell culture supernatants would be increased in response to a PAR-1 peptide agonist. Cells were pre-treated with the COX inhibitor indomethacin, in order to determine whether the effect of PAR-1 on coagulation factor release was mediated *via* prostanoid production.

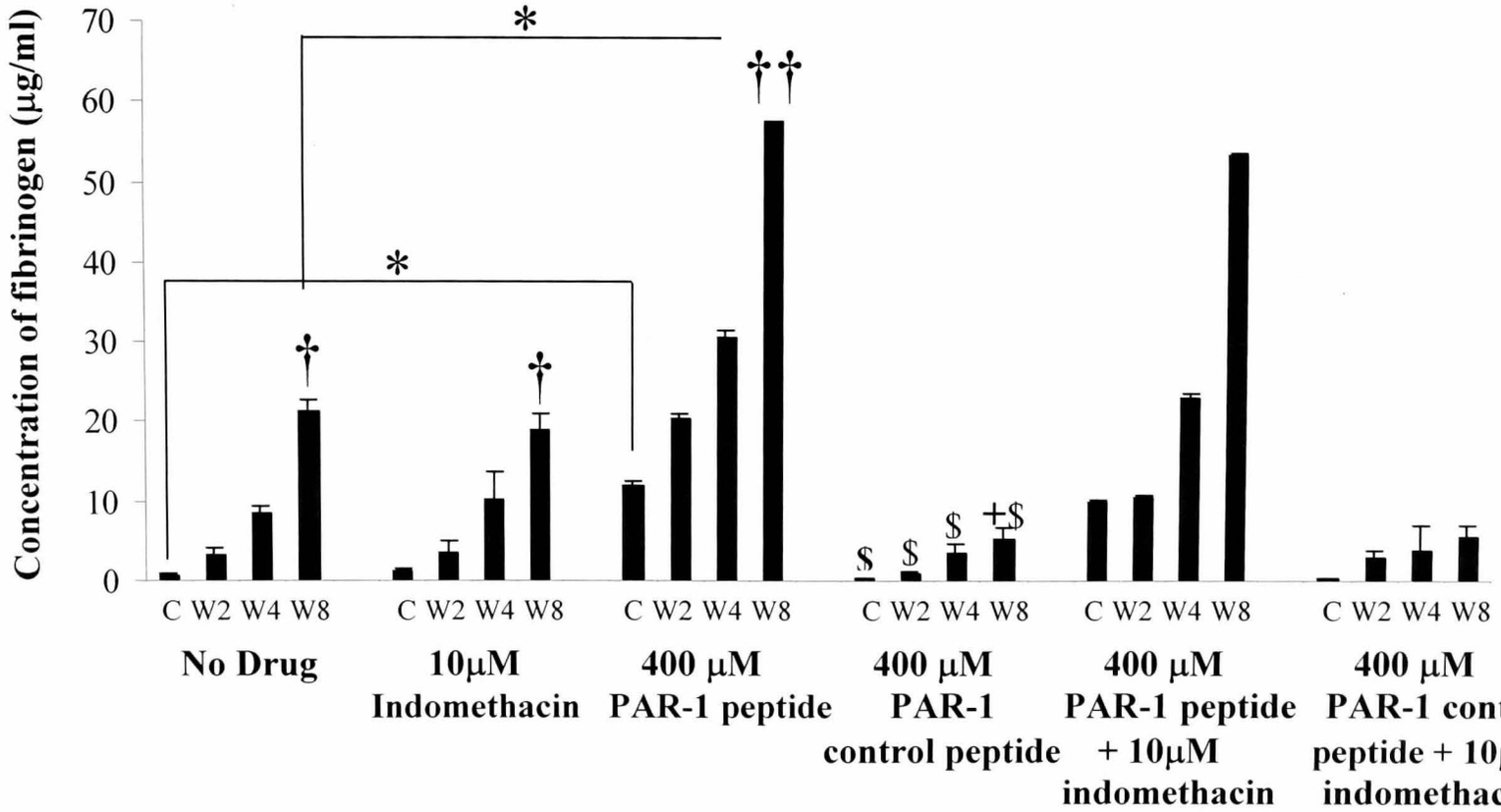

Figure 5.6. Effect of mechanical wounding, PAR-1 peptide agonists and indomethacin on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours post-wounding. C=unwounded, W2=2 wounds, W4=4 wounds & W8=8 wounds. † Indicates $P<0.05$; †† indicates $P<0.005$ compared to C and * indicates $P<0.005$; no drug versus 400 µM PAR-1 control peptide: + indicates $P<0.005$; 400 µM PAR-1 peptide versus 400 µM PAR-1 control peptide: $ indicates $P<0.0005$. Data represent mean ± SEM (*n=3*).

Figure 5.6 indicates the effect of a PAR-1 peptide agonist in the absence and presence of indomethacin on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours *post*-wounding.

In the absence of the PAR-1 peptide agonist, there was a significant increase in the concentration of fibrinogen in cell culture supernatants with the degree of wounding: W2 = 3.18 ± 1.47 μg/ml; W4 = 8.41 ± 3.45 μg/ml and W8 = 21.23 ± 1.42 μg/ml compared to the unwounded control, C = 0.67 ± 0.17 μg/ml, confirming the results presented in *section 3.4.2.1.*

Indomethacin is a non-specific COX inhibitor and was used to investigate whether the effects of wounding on fibrinogen release was mediated *via* the release of prostanoids. Pre-incubation of cells with indomethacin had no significant effect on the concentration of fibrinogen in cell culture supernatants compared to the wounded controls.

The synthetic PAR-1 peptide agonist, TFRIFD-amide enhanced the concentration of fibrinogen in cell culture supernatants compared to the no PAR-1 peptide agonist control. Levels of fibrinogen in *un*wounded cell culture supernatants were significantly higher in cell cultures stimulated with PAR-1 (C = 12.02 ± 0.40 μg/ml) compared to the no PAR-1 peptide agonist control (C = 0.67 ± 0.17 μg/ml). The presence of the PAR-1 peptide agonist increased the concentration of fibrinogen in cell culture supernatants with the degree of wounding (W2 = 20.28 ± 0.69 μg/ml and W4 = 30.65 ± 0.99 μg/ml) and with maximal wounding, PAR-1 significantly enhanced the concentration of fibrinogen in cell culture supernatants (W8 = 58.24 ± 0.45 μg/ml) compared to the no PAR-1 peptide agonist control (W8 = 21.23 ± 1.42 μg/ml).

The synthetic scrambled PAR-1 control peptide agonist, FTRIFD-amide had no significant effect on the levels of fibrinogen in supernatants of unwounded 16HBE 14o$^-$ cells and those denoted by W2 and W4. However, the control peptide significantly reduced the levels of fibrinogen in supernatants (W8 = 5.32 ± 0.48 μg/ml) compared to the no drug control (W8 = 21.23 ± 1.33 μg/ml).

Interestingly, the PAR-1 control peptide agonist significantly reduced the levels of fibrinogen in cell culture supernatants (C = 0.22 ± 0.01 μg/ml; W2 = 0.91 ± 0.03 μg/ml; W4 = 3.54 ± 0.21 μg/ml and W8 = 5.32 ± 0.48 μg/ml) compared to the PAR-1 peptide agonist (C = 12.02 ± 1.18 μg/ml; W2 = 20.28 ± 1.75 μg/ml; W4 = 30.65 ± 2.09 μg/ml and W8 = 58.24 ± 4.31 μg/ml).

The presence of 10 μM indomethacin had no effect on the response to the PAR-1 peptide agonist, suggesting that the effects of PAR-1 on the release of fibrinogen from 16HBE 14o$^-$ cells is not mediated *via* prostanoids.

Similarly, the presence of 10 μM indomethacin had no effect on the response to the scrambled PAR-1 control peptide agonist.

5.4.2.2. Effect of PAR-2

Since the activation of PAR-2 by a PAR-2 peptide agonist enhanced repair (*section 5.4.1.2*), it was of interest to investigate the effects of PAR-2 activation on the release of coagulation factors into culture supernatants of 16HBE 14o$^-$ cells. If wound repair was occurring *via* fibrin formation as a result of PAR-2 activation, it was expected that the concentration of coagulation factors and D-dimers would be increased in response to a PAR-2 peptide agonist. Because PAR-2 activation is known to elicit a bronchoprotective response, which is mediated *via* PGE_2, the PAR-2 peptide agonist was added in the absence and presence of the COX inhibitor indomethacin, in order to determine whether the effect of PAR-2 on coagulation factor release was mediated *via* PGE_2.

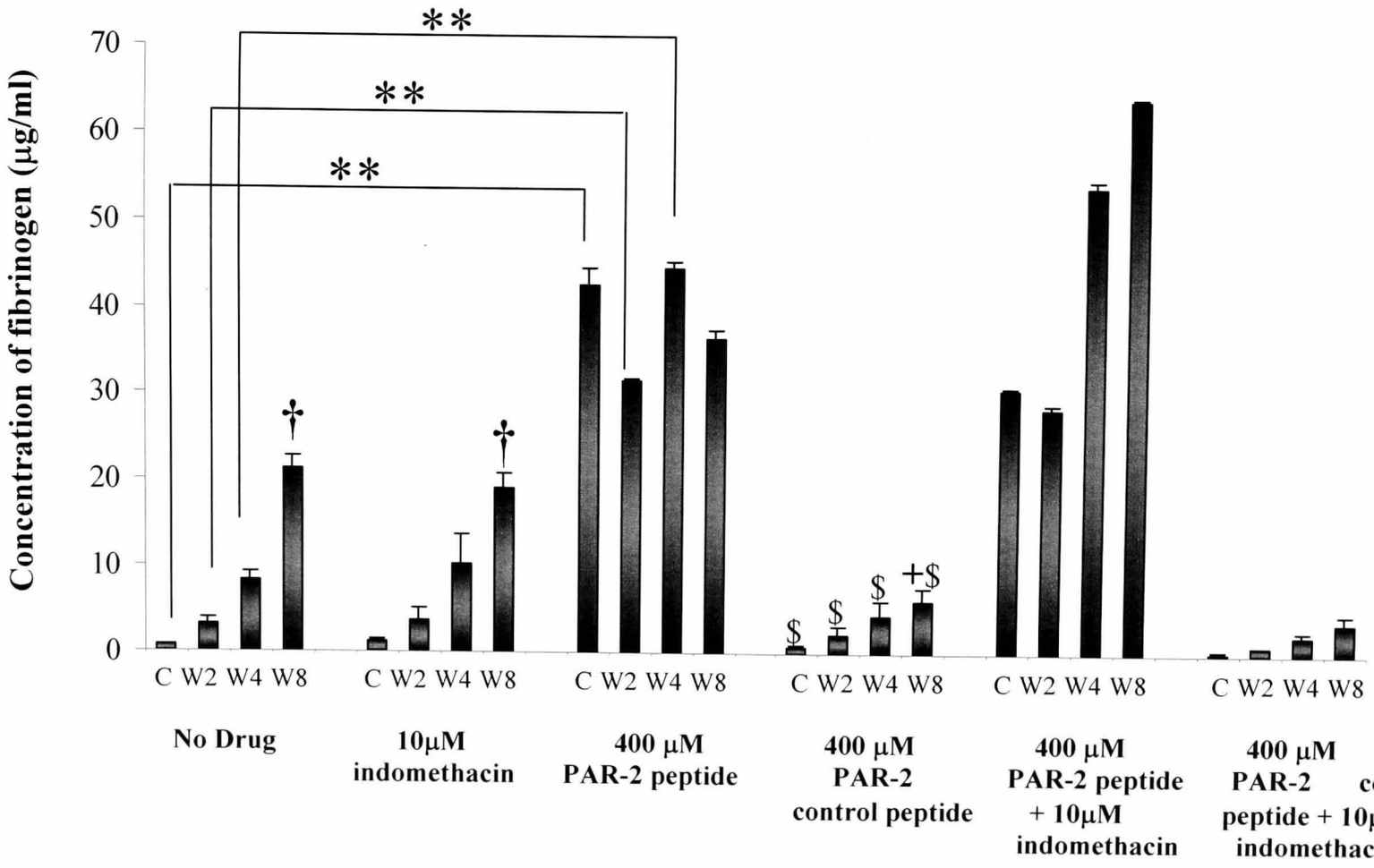

Figure 5.7. Effect of mechanical wounding, PAR-2 peptide agonists and indomethacin on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours post-wounding. C=unwounded, W2=2 wounds, W4=4 wounds & W8=8 wounds. † Indicates $P<0.05$ compared to C and ** indicates $P<0.02$; no drug versus 400 µM PAR-2 control peptide: + indicates $P<0.05$; 400 µM PAR-2 peptide versus 400 µM PAR-2 control peptide: $ indicates $P<0.005$. Data represent mean ± SEM ($n=3$).

Figure 5.7 indicates the effect of a PAR-2 peptide agonist in the absence and presence of indomethacin on the concentration of fibrinogen in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours *post*-wounding.

In the absence of the PAR-2 peptide agonist, there was a significant increase in the concentration of fibrinogen in cell culture supernatants with the degree of wounding: W2 = 3.18 ± 1.47 μg/ml, W4 = 8.41 ± 3.45 μg/ml and W8 = 21.23 ± 1.42 μg/ml compared to the unwounded control, C = 0.67 ± 0.17 μg/ml confirming the results presented in *section 3.4.2.1.*

Addition of the COX inhibitor indomethacin to the medium had no significant effect on the release of fibrinogen into culture supernatants of 16HBE 14o$^-$ cells compared to the wounded controls.

The synthetic PAR-2 peptide agonist, SLIGKVD-amide significantly enhanced the concentration of fibrinogen in cell culture supernatants compared to the no PAR-2 peptide agonist control. This was particularly evident at baseline, whereby the concentration of fibrinogen in *un*wounded cell culture supernatants of cell cultures stimulated with PAR-2 were 42.63 ± 1.91 μg/ml compared to 0.67 ± 0.17 μg/ml in the no PAR-2 peptide agonist control. In the wounded cell cultures, the PAR-2 peptide agonist significantly enhanced the concentration of fibrinogen in cell culture supernatants (W2 = 31.58 ± 0.35 μg/ml; W4 = 44.59 ± 0.67 μg/ml and W8 = 36.52 ± 1.04 μg/ml) compared to the no PAR-2 peptide agonist control (W2 = 3.18 ± 1.47 μg/ml; W4 = 8.41 ± 3.45 μg/ml and W8 = 21.23 ± 1.42 μg/ml). With respect to the effect of the PAR-2 peptide agonist alone, there was no further increase in the concentration of fibrinogen in cell culture supernatants with the degree of wounding.

With respect to the *un*wounded cell cultures, the effect of the PAR-2 peptide agonist on supernatant concentration of fibrinogen was significantly greater ($P<0.005$) than that of PAR-1.

The synthetic scrambled PAR-2 control peptide agonist, LSIGKVD-amide had no significant effect on the levels of fibrinogen in supernatants of unwounded 16HBE 14o-

cells and those denoted by W2 and W4. However, the control peptide significantly reduced the levels of fibrinogen in supernatants denoted by W8 = 6.15 ± 0.31 μg/ml compared to the no drug control (W8 = 21.23 ± 1.33 μg/ml).

Interestingly, the PAR-2 control peptide agonist significantly reduced the levels of fibrinogen in cell culture supernatants (C = 0.76 ± 0.64 μg/ml; W2 = 2.22 ± 0.24 μg/ml; W4 = 4.33 ± 0.32 μg/ml and W8 = 6.15 ± 0.31 μg/ml) compared to the PAR-2 peptide agonist (C = 42.63 ± 3.14 μg/ml; W2 = 31.58 ± 1.96 μg/ml; W4 = 44.59 ± 1.86 μg/ml and W8 = 36.52 ± 2.90 μg/ml).

The presence of 10 μM indomethacin had no effect on the response to the PAR-2 peptide agonist, suggesting that the effects of PAR-2 on the release of fibrinogen into cell culture supernatants are not mediated *via* PGE_2.

Similarly, the presence of 10 μM indomethacin had no effect on the response to the scrambled PAR-2 control peptide agonist.

5.4.3. Concentration of FXIIIA in cell culture supernatants

5.4.3.1. Effect of PAR-1

Figure 5.8 demonstrates the effect of the PAR-1 peptide agonist on the concentration of FXIIIA in cell culture supernatants 2 hours *post*-wounding. In order to determine whether the effects of PAR-1 activation were mediated *via* prostanoids, the PAR-1 peptide agonist was added in the absence and presence of the COX inhibitor indomethacin.

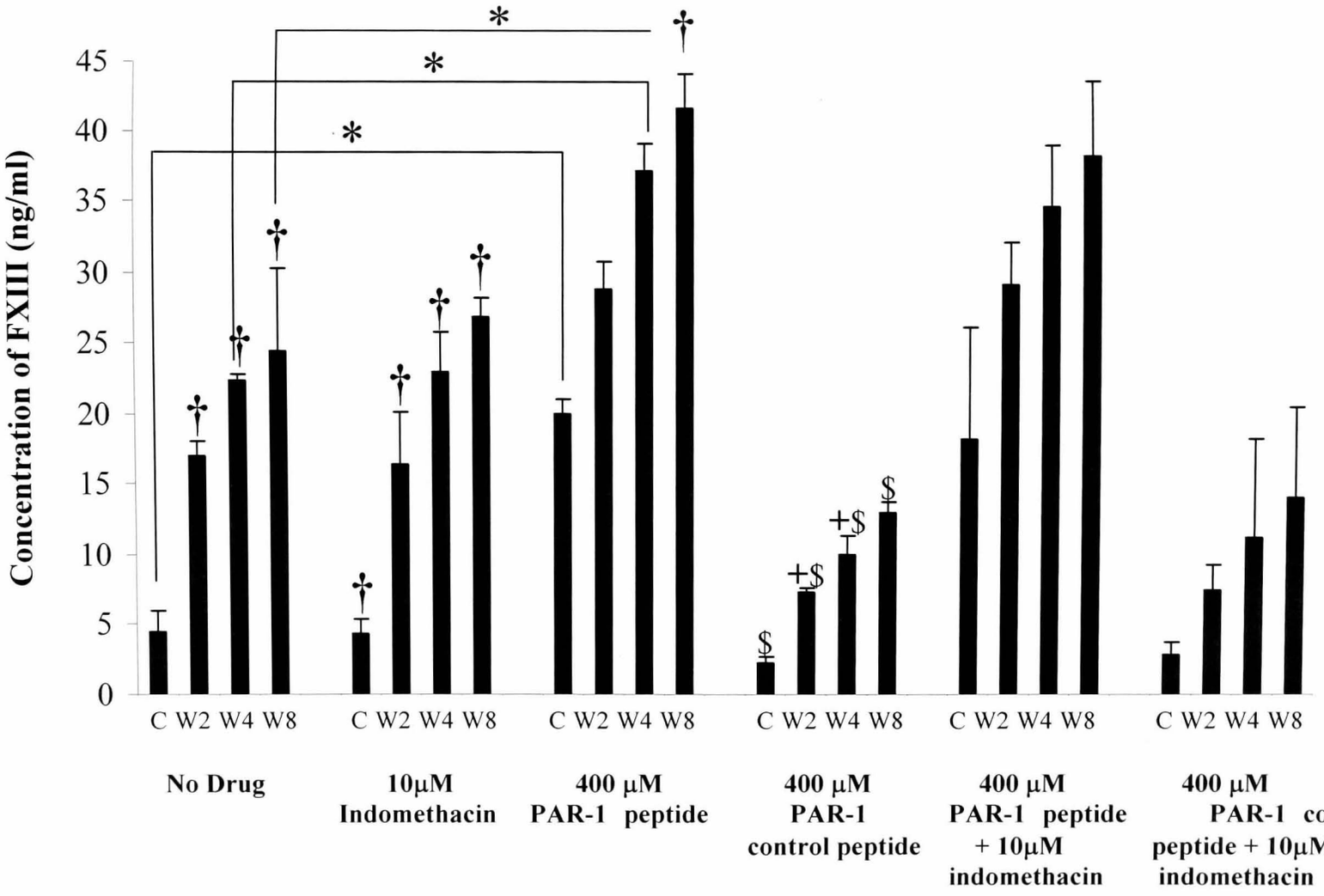

Figure 5.8. Effect of mechanical wounding, PAR-1 peptide agonists and indomethacin on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours *post*-wounding. C=unwounded, W2=2 wounds, W4=4 wounds & W8=8 wounds. † Indicates $P<0.05$ compared to C and * indicates $P<0.05$; no drug versus 400 μM PAR-1 control peptide: + indicates $P<0.05$; 400 μM PAR-1 peptide versus 400 μM PAR-1 control peptide: $ indicates $P<0.0005$. Data represent mean ± SEM (*n=3*).

In the absence of the PAR-1 peptide agonist, there was a significant increase in the concentration of FXIIIA in cell culture supernatants with the degree of wounding: W2 = 17.00 ± 1.06 ng/ml; W4 = 22.30 ± 0.50 ng/ml and W8 = 24.43 ± 5.87 ng/ml compared to the unwounded control, C = 4.49 ± 1.50 ng/ml, as observed in previous experiments (*section 3.4.2.2*).

Addition of the COX inhibitor indomethacin to the medium had no significant effect on the concentration of FXIIIA in cell culture supernatants compared to the wounded controls.

The synthetic PAR-1 peptide agonist, TFRIFD-amide significantly enhanced the concentration of FXIIIA in *un*wounded and wounded cell culture supernatants (C = 19.97 ± 1.07 ng/ml; W2 = 28.72 ± 1.93 ng/ml; W4 = 37.14 ± 1.85 ng/ml and W8 = 41.57 ± 2.52 ng/ml) compared to the no PAR-1 peptide agonist controls (C = 4.49 ± 1.50 ng/ml, W2 = 17.00 ± 1.06 ng/ml; W4 = 22.30 ± 0.50 ng/ml and W8 = 24.43 ± 5.87 ng/ml). With respect to the effects of the PAR-1 peptide agonist alone, there was an increase in the concentration of FXIIIA in cell culture supernatants with the extent of wounding.

The synthetic scrambled PAR-1 control peptide agonist, FTRIFD-amide significantly reduced the levels of FXIIIA in supernatants (W2 = 7.30 ± 0.22 μg/ml and W4 = 10.03 ± 0.75 μg/ml) compared to the no drug control (W2 = 17.00 ± 1.52 μg/ml and W4 = 22.30 ± 2.40 μg/ml).

Interestingly, the PAR-1 control peptide agonist significantly reduced the levels of FXIIIA in cell culture supernatants (C = 2.18 ± 0.08 μg/ml; W2 = 7.30 ± 0.22 μg/ml: W4 = 10.03 ± 0.75 μg/ml and W8 = 12.93 ± 0.42 μg/ml) compared to the PAR-1 peptide agonist (C = 19.97 ± 1.35 μg/ml; W2 = 28.72 ± 1.65 μg/ml; W4 = 37.14 ± 1.48 μg/ml and W8 = 41.57 ± 1.88 μg/ml).

The presence of 10 μM indomethacin had no effect on the response to PAR-1, suggesting that the effects of PAR-1 on the concentration of FXIIIA in cell culture supernatants are not mediated *via* prostanoids.

Similarly, the presence of 10 μM indomethacin had no effect on the response to the scrambled PAR-1 control peptide agonist.

5.4.3.2. PAR-2

Figure 5.9 demonstrates the effect of the PAR-2 peptide agonist on the concentration of FXIIIA in cell culture supernatants 2 hours *post*-wounding. In order to determine whether the effects of PAR-2 activation were mediated *via* PGE_2, the PAR-2 peptide agonist was added in the absence and presence of the COX inhibitor indomethacin.

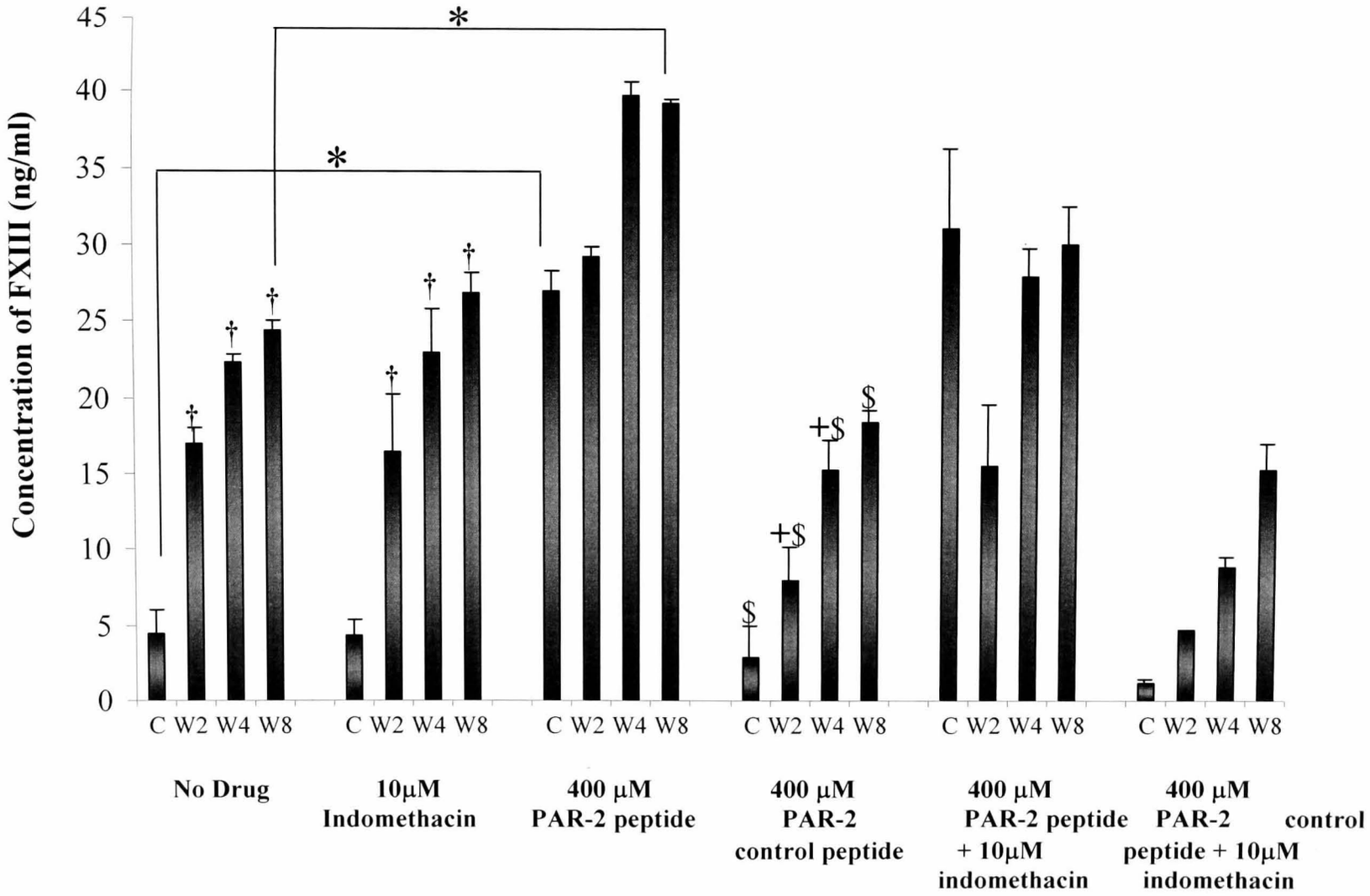

Figure 5.9. Effect of mechanical wounding, PAR-2 peptide agonists and indomethacin on the concentration of FXIIIA in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours *post*-wounding. C=unwounded, W2=2 wounds, W4=4 wounds & W8=8 wounds. † Indicates $P<0.05$ compared to C and * indicates $P<0.05$; no drug versus 400 μM PAR-2 control peptide: + indicates $P<0.05$; 400 μM PAR-2 peptide versus 400 μM PAR-2 control peptide: $ indicates $P<0.0005$. Data represent mean ± SEM (*n=3*).

In the absence of the PAR-2 peptide agonist, there was a significant increase in the concentration of FXIIIA in cell culture supernatants with the degree of wounding: W2 = 17.00 ± 1.06 ng/ml; W4 = 22.30 ± 0.50 ng/ml and W8 = 24.43 ± 5.87 ng/ml compared to the unwounded control, C = 4.49 ± 1.50 ng/ml, as observed in previous experiments (*section 3.4.2.2*).

Addition of the COX inhibitor indomethacin to the medium had no significant effect on the concentration of FXIIIA in cell culture supernatants compared to the wounded controls.

The synthetic PAR-2 peptide agonist, SLIGKVD-amide enhanced the concentration of FXIIIA in cell culture supernatants (C = 27.05 ± 1.20 ng/ml; W2 = 29.26 ± 0.63 ng/ml; W4 = 39.72 ± 1.00 ng/ml and W8 = 39.25 ± 0.25 ng/ml) compared to the no PAR-2 peptide agonist control (C = 4.49 ± 1.50 ng/ml, W2 = 17.00 ± 1.06 ng/ml, W4 = 22.30 ± 0.50 ng/ml and W8 = 24.43 ± 5.87 ng/ml) and its effects were significant in the unwounded cell cultures and in those depicted by W4. With respect to the effects of the PAR-2 peptide agonist alone, there was an increase in the concentration of FXIIIA in cell culture supernatants with the degree of wounding, however, this effect was not quite significant (P=0.072).

The synthetic scrambled PAR-2 control peptide agonist, LSIGKVD-amide significantly reduced the levels of FXIIIA in supernatants (W2 = 7.92 ± 1.26 μg/ml and W4 = 15.23 ± 0.65 μg/ml) compared to the no drug control (W2 = 17.00 ± 1.52 μg/ml and W4 = 22.30 ± 2.40 μg/ml).

Interestingly, the PAR-2 control peptide agonist significantly reduced the levels of FXIIIA in cell culture supernatants (C = 2.92 ± 0.25 μg/ml; W2 = 7.92 ± 1.26 μg/ml; W4 = 15.23 ± 0.65 μg/ml and W8 = 18.39 ± 0.59 μg/ml) compared to the PAR-2 peptide agonist (C = 19.97 ± 1.35 μg/ml; W2 = 29.26 ± 0.87 μg/ml; W4 = 39.72 ± 0.95 μg/ml and W8 = 39.25 ± 2.02 μg/ml).

The presence of 10 μM indomethacin had no effect on the response to PAR-2 suggesting that the effects of PAR-2 on the concentration of FXIIIA in cell culture supernatants are not mediated *via* PGE_2.

Similarly, the presence of 10 μM indomethacin had no effect on the response to the scrambled PAR-2 control peptide agonist.

5.4.4. Expression of D-dimers

5.4.4.1. PAR-1

Figure 5.10 demonstrates the effect of the PAR-1 peptide agonist on the concentration of D-dimers, the marker of fibrin formation and fibrin breakdown in cell culture supernatants 2 hours *post*-wounding. In order to determine whether the effects of PAR-1 activation were mediated *via* prostanoids, the PAR-1 peptide agonist was added in the absence and presence of the COX inhibitor indomethacin.

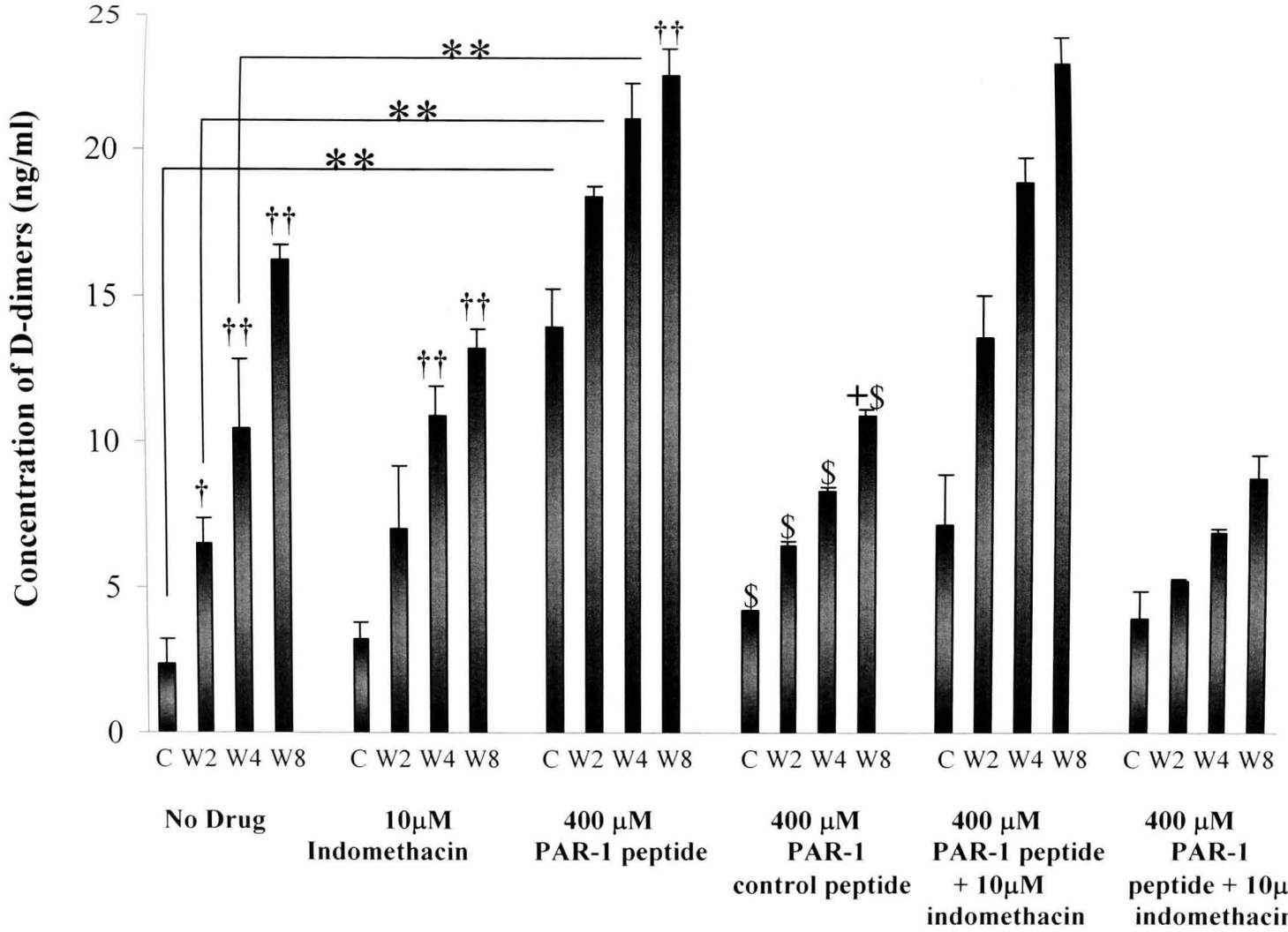

Figure 5.10. Effect of mechanical wounding, PAR-1 peptide agonists and indomethacin on the concentration of D-dimers in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours post-wounding. C=unwounded, W2=2 wounds, W4=4 wounds & W8=8 wounds. † Indicates $P<0.05$ †† indicates $P<0.02$ compared to C and ** indicates $P<0.02$; no drug versus 400 μM PAR-1 control peptide: + indicates $P<0.05$; 400 μM PAR-1 peptide versus 400 μM PAR-1 control peptide: $ indicates $P<0.005$. Data represent mean ± SEM (*n=3*).

In the absence of PAR-1 peptide agonist, there was a significant increase in the concentration of D-dimers in cell culture supernatants with the degree of wounding: W2 = 6.54 ± 0.85 ng/ml; W4 = 10.42 ± 2.39 ng/ml and W8 = 16.21 ± 0.49 ng/ml, compared to the unwounded control, C = 2.33 ± 0.88 ng/ml; indicative of fibrin turnover, confirming the results presented in *section 3.4.2.3*.

Addition of the COX inhibitor indomethacin to the medium had no effect on the concentration of D-dimers in cell culture supernatants compared to the wounded controls.

The synthetic PAR-1 peptide agonist, TFRIFD-amide significantly enhanced the concentration of D-dimers in cell culture supernatants (C = 13.87 ± 1.35 ng/ml; W2 = 18.34 ± 0.36 ng/ml; W4 = 21.05 ± 1.32 ng/ml and W8 = 22.64 ± 1.03 ng/ml) compared to the no PAR-1 peptide agonist control (C = 2.33 ± 0.88 ng/ml; W2 = 6.54 ± 0.85 ng/ml; W4 = 10.42 ± 2.39 ng/ml and W8 = 16.21 ± 0.49 ng/ml). With respect to the effects of the PAR-1 peptide agonist alone, there was an increase in the concentration of D-dimers in cell culture supernatants with the degree of wounding.

The synthetic scrambled PAR-1 control peptide agonist, FTRIFD-amide had no significant effect on the levels of D-dimers in supernatants of unwounded 16HBE 14o- cells and those denoted by W2 and W4. However, the control peptide significantly reduced the levels of D-dimers in supernatants (W8 = 10.89 ± 0.75 μg/ml) compared to the no drug control (W8 = 16.21 ± 1.10 μg/ml).

Interestingly, the PAR-1 control peptide agonist significantly reduced the levels of D-dimers in cell culture supernatants (C = 4.19 ± 1.32 μg/ml; W2 = 6.46 ± 1.17 μg/ml; W4 = 8.34 ± 0.95 μg/ml and W8 = 10.89 ± 0.75 μg/ml) compared to the PAR-1 peptide agonist (C = 13.87 ± 2.16 μg/ml; W2 = 18.34 ± 1.28 μg/ml; W4 = 21.05 ± 2.26 μg/ml and W8 = 22.64 ± 0.62 μg/ml).

The presence of 10 μM indomethacin had no effect on the response to PAR-1, suggesting that the effects of PAR-1 on the concentration of D-dimers in cell culture supernatants are not mediated *via* prostanoids.

Similarly, the presence of 10 μM indomethacin had no effect on the response to the scrambled PAR-1 control peptide agonist.

5.4.4.2. PAR-2

Figure 5.11 demonstrates the effect of the PAR-2 peptide agonist on the concentration of D-dimers, the marker of fibrin formation and breakdown, in cell culture supernatants 2 hours *post*-wounding. In order to determine whether the effects of PAR-2 activation were mediated *via* PGE_2, the PAR-2 peptide agonist was added in the absence and presence of the COX inhibitor indomethacin.

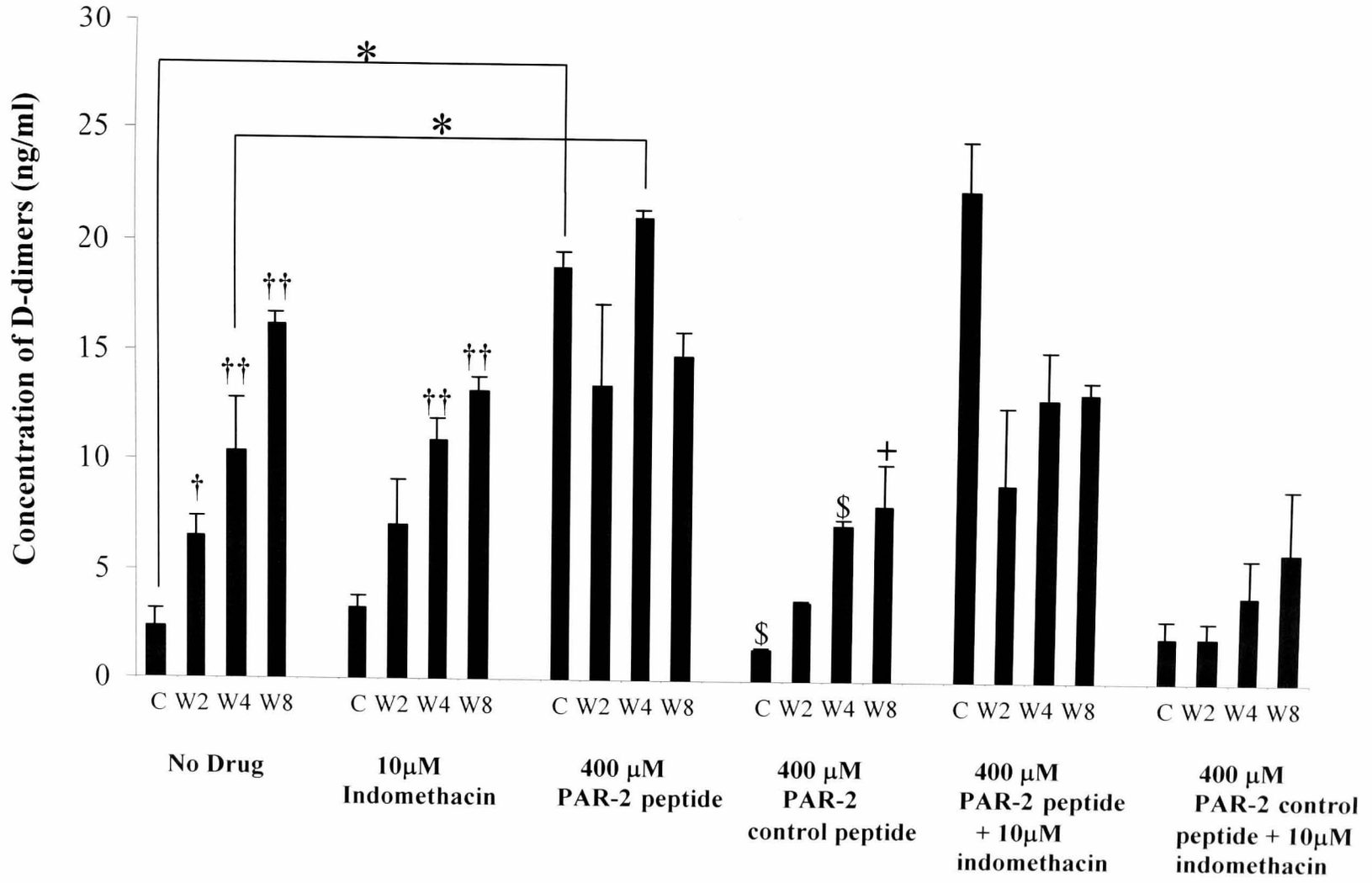

Figure 5.11. Effect of mechanical wounding, PAR-2 peptide agonists and indomethacin on the concentration of D-dimers in cell culture supernatants of 16HBE 14o$^-$ cells 2 hours post-wounding. C=unwounded, W2=2 wounds, W4=4 wounds & W8=8 wounds. † Indicates $P<0.05$ †† indicates $P<0.02$ compared to C and * indicates $P<0.05$; no drug versus 400 μM PAR-2 control peptide: + indicates $P<0.05$; 400 μM PAR-2 peptide versus 400 μM PAR-2 control peptide: $ indicates $P<0.0005$. Data represent mean ± SEM (*n=3*).

In the absence of PAR-2 peptide agonist, there was a significant increase in the concentration of D-dimers in cell culture supernatants with the degree of wounding: W2 = 6.54 ± 0.85 ng/ml; W4 = 10.42 ± 2.39 ng/ml and W8 = 16.21 ± 0.49 ng/ml compared to the unwounded control, C = 2.33 ± 0.88 ng/ml, confirming the results presented in *section 3.4.2.3*.

Addition of the COX inhibitor indomethacin to the medium had no significant effect on the concentration of D-dimers in cell culture supernatants compared to the wounded controls.

The synthetic PAR-2 peptide agonist, SLIGKVD-amide significantly enhanced the concentration of D-dimers in cell culture supernatants compared to the no PAR-2 peptide agonist control at baseline, whereby the concentration of D-dimers in cell culture supernatants of cell cultures stimulated with PAR-2 was 18.81 ± 0.67 ng/ml compared to 2.33 ± 0.88 ng/ml in the no PAR-2 peptide agonist control. In the wounded cell cultures, the PAR-2 peptide agonist enhanced the concentration of D-dimers in cell culture supernatants (W2 = 13.46 ± 3.74 ng/ml and W4 = 21.09 ± 0.31 ng/ml) compared to the no PAR-2 peptide agonist control (W2 = 6.54 ± 0.85 ng/ml and W4 = 10.42 ± 2.39 ng/ml); however, these effects were only significant with regards to W4. With respect to the effect of the PAR-2 peptide agonist alone, there was no change in the concentration of D-dimers in cell culture supernatants with the degree of wounding.

The synthetic scrambled PAR-2 control peptide agonist, LSIGKVD-amide had no significant effect on the levels of D-dimers in supernatants of unwounded 16HBE 14o$^-$ cells and those denoted by W2 and W4. However, the control peptide significantly reduced the levels of D-dimers in supernatants (W8 = 9.20 ± 1.34 μg/ml) compared to the no drug control (W8 = 16.21 ± 1.10 μg/ml).

Interestingly, the PAR-2 control peptide agonist significantly reduced the levels of D-dimers in supernatants of unwounded cell cultures (C = 2.94 ± 0.60 μg/ml) and those denoted by W4 = 7.36 ± 0.84 μg/ml compared to the PAR-2 peptide agonist (C = 18.81 ± 0.99 μg/ml and W4 = 21.09 ± 1.23 μg/ml).

The presence of 10 μM indomethacin had no effect on the response to PAR-2, suggesting that the effects of PAR-2 on the concentration of D-dimers in cell culture supernatants are not mediated *via* PGE_2.

Similarly, the presence of 10 μM indomethacin had no effect on the response to the scrambled PAR-2 control peptide agonist.

5.5. Summary of results

Activation of both PAR-1 and PAR-2 significantly enhanced the rate of wound repair of 16HBE 14o$^-$ cells and the effect of PAR-2 was demonstrated to be significantly greater than that of PAR-1. With respect to the data generated from immunoblotting, activation of PAR-1 and PAR-2 induced a significant increase in the release of fibrinogen, FXIIIA and D-dimers from 16HBE 14o$^-$ cells at baseline and from wounded monolayers. Indomethacin had no significant effect on the release of fibrinogen, FXIII or D-dimers in response to either PAR-1 or PAR-2 activation.

5.6. Discussion

With respect to data generated from immunoblotting, wounding alone stimulated an increase in the release of fibrinogen, FXIIIA and D-dimers from 16HBE 14o$^-$ cells indicative of sequential fibrinogenesis and fibrinolysis as previously demonstrated in independent experiments described in *Chapter 3*. The COX inhibitor indomethacin had no effect on the observed response indicating that PGE_2 was not involved. The lack of involvement of PGE_2 is in accordance with evidence for the limited production of this prostanoid under the current culture conditions and the lack of EP-1 and EP-4 expression by 16HBE 14o$^-$ cells (*Chapter 6*).

PAR-1 and PAR-2 are expressed by the bronchial epithelium (Cocks *et al.*, 1999; Cocks *et al.*, 2000; Lan *et al.*, 2002). Expression of both receptors has previously been demonstrated in 16HBE 14o$^-$ cells (Page *et al.*, 2003) and their expression in this cell line has been confirmed (*Chapter 6*). Unlike conventional receptors that bind diffuse, soluble ligands, PARs are activated *via* a highly unique and specific mechanism. PARs

require specific site-directed amino-terminal proteolysis by select serine proteases to be self activated by newly formed amino-terminals, termed 'tethered ligands' (Cocks *et al.*, 2000).

Synthetic peptide analogues that mimic a portion of the amino acid sequence of the PAR-tethered ligand are routinely used to activate PARs -1, -2 and -4, but interestingly, not PAR-3. These agonists are reported to have greater selectivity for PARs than do the endogenous ligands such as trypsin and thrombin. However, they display a low potency, hence the requirement for high concentrations to activate the receptor (Lan *et al.*, 2002). A previous study determined the specificity of PAR peptide agonists using xenopus oocytes. PAR-1 did not respond to trypsin or the human PAR-2 peptide agonist SLIGKVD, whereas PAR-2 was demonstrated to respond to the human PAR-1 peptide SFLLRN-amide but not to TFRIFD-amide, the xenopus PAR-1 peptide agonist. TFRIFD-amide activated PAR-1 with an EC_{50} of 1 μM but was unable to activate PAR-2 at concentrations up to 50 μM (Blackhart *et al.*, 1996). The genes for PAR-1 and PAR-2 are both located on chromosome 5, therefore it is unsurprising that these receptors share a similar amino acid sequence and in terms of the second extracellular loop, which binds the tethered ligand, the identity is approximately 72% homologous (Blackhart *et al.*, 1996), giving rise to the possibility of ligand cross-reactivity.

In view of the report by Blackhart *et al.* that demonstrated ligand cross-reactivity, the synthetic xenopus peptide agonist TFRIFD-amide and human SLIGKVD-amide were employed in the current study to activate PAR-1 and PAR-2 respectively. And corresponding partially scrambled peptides (FTRIFD-amide and LSIGKVD-amide) were included to verify PAR activation. The same PAR peptide agonists were previously used to activate PAR-1 and PAR-2 on 16HBE 14o- cells and to demonstrate PAR-2 specific effects on IL-8 expression (Page *et al.*, 2003). The peptide concentration of 400 μM was the same as that employed previously to activate PAR-1 and PAR-2 on human bronchial epithelial cells (Asokananthan *et al.*, 2002). The specificity of scrambled PAR peptide agonists has been reported to be questionable in some assay systems. In a bioassay to determine the role of PAR-4, scrambled peptide agonist controls were demonstrated to exert biological effects (Moffatt, 2004).

Activation of both PAR-1 and PAR-2 by synthetic peptide agonists significantly enhanced the release of fibrinogen and FXIIIA from 16HBE 14o$^-$ cells at baseline and from wounded monolayers. This response was not inhibited by indomethacin, indicating that PGE_2 was not involved in the observed response. Activation of PAR-1 was previously demonstrated to stimulate the release of FXIII from chondrocytes (Rosenthal *et al.*, 2004). The fact that PAR activation leads to the release of these coagulation factors signifies that following wounding of unstimulated 16HBE 14o$^-$ monolayers, the repair response may involve initiation of the coagulation cascade and/or activation of PARs.

The level of D-dimer release from 16HBE 14o$^-$ cells at baseline and from wounded monolayers was significantly enhanced as a result of PAR-1 and PAR-2 activation. Since D-dimers are indicative of fibrin formation and subsequent degradation, this implies that the increased release of fibrinogen and FXIIIA following PAR activation lead to an enhancement of coagulation that did not involve PGE_2. The presence of D-dimers provides evidence that fibrinolysis is occurring in the current model. This suggests that t-PA and plasminogen are present in the cell culture for the generation of plasmin that mediates fibrinolysis.

With respect to the wounded cultures, in particular that denoted by 'W8' indicating maximal wounding, the effect of PAR-1 on the release of D-dimers was greater than that of PAR-2, although the difference was not significant (P=0.14). However, it is possible that PAR-1 is more important in fibrinolysis.

The partially scrambled PAR-1 and PAR-2 control peptide agonists appeared to have a biological effect in the current model as they each demonstrated a significant reduction in the levels of fibrinogen, FXIIIA and D-dimers compared to both the no drug control and the corresponding PAR peptide. This may be indicative of a potential antagonistic effect. However, they did not demonstrate any effect in the wound repair assay.

Activation of PAR receptors by peptide agonists of PAR-1 and PAR-2 was also demonstrated to significantly enhance the rate of wound repair. Conceivably, wound repair may have occurred *via* the release of PGE_2 (Asokananthan *et al.*, 2002) or coagulation factor release and subsequent fibrin formation. PAR-2 activation was

previously shown to facilitate wound healing by release of the secondary mediator PGE_2 from the epithelium (Cocks *et al.*, 2000). Given that the current culture conditions limit the production of this prostanoid, it is most likely that PAR-mediated wound repair involved the release of coagulation factors and subsequent fibrin formation, independently of PGE_2 production. Interestingly, the effect of PAR-2 activation on wound repair was determined to be significantly greater than that of PAR-1 and PAR-2 was also shown to have a significantly greater effect on the release of fibrinogen compared to that of PAR-1 suggesting that PAR-2 is more important in fibrin formation and wound repair.

PAR-1 activation is known to elicit an array of acute signalling responses including phosphoinositide hydrolysis, calcium mobilisation, PKC activation, ERK activation and c-*fos* expression, which are required for protein synthesis and protein tyrosine phosphorylation, which is required for thrombin-dependent mitogenesis (Berk *et al.*, 1991; Seuwen *et al.*, 1990).

PAR-1 activation by thrombin has been demonstrated to transactivate EGFR and increase EGFR phosphorylation (*figure 5.12*) (Sabri *et al.*, 2002). More recently, PAR-1 has been shown to mediate the tyrosine phosphorylation of EGFR in human renal carcinoma cells (Bergmann *et al.*, 2006).

Previous *in vitro* studies have demonstrated that EGF stimulates IL-8 release from primary NHBE cells *via* activation of EGFR (Hamilton *et al.*, 2003). The release of IL-8 from 16HBE $14o^-$ cells in response to wounding was demonstrated in the current study (*section 3.4.6*). Thus, the fact that PAR activation is reported to stimulate the release of IL-8 suggests the involvement of EGFR in the observed response.

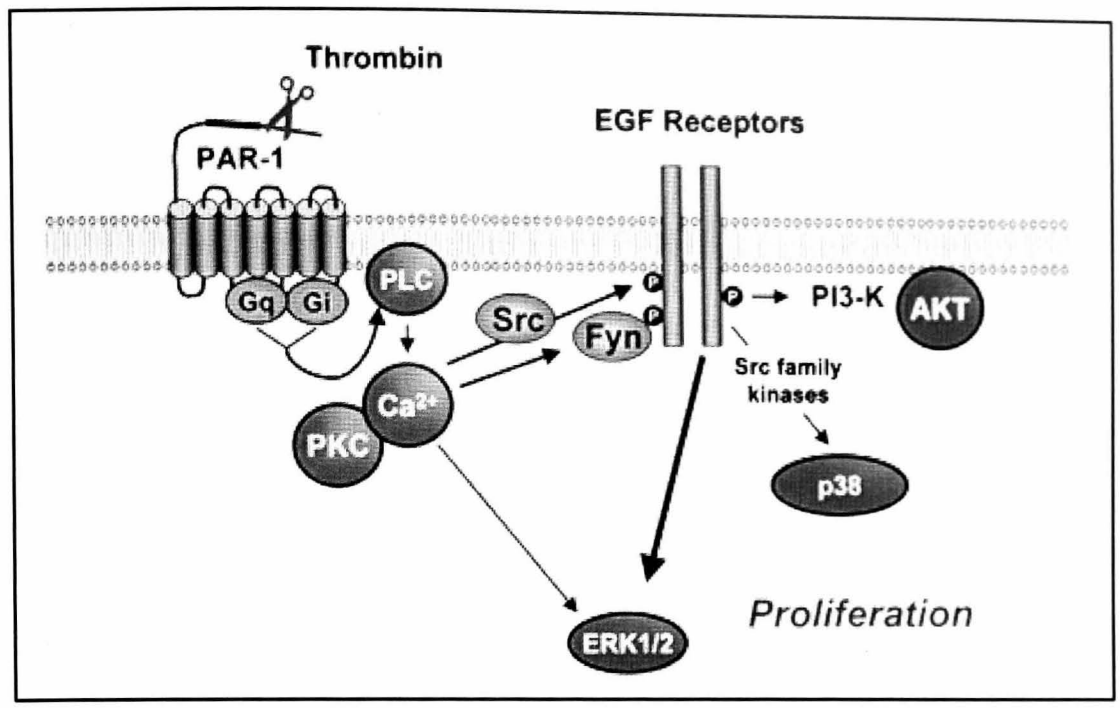

Figure 5.12. Mechanism of PAR-1 signalling.
PAR-1 couples to G proteins, leading to rapid activation of PLC and subsequent formation of IP_3 and mobilisation of intracellular calcium. Diacylglycerol and PKC activation are predicted to occur in parallel. Thrombin also activates Src family kinases. Both Src and Fyn are activated, which in turn associate with EGFR and increase phosphorylation (Sabri *et al.*, 2002).

Activation of PAR-2 on epithelial cells has been demonstrated to induce the release of inflammatory mediators such as: IL-6, IL-8, MMP-9 and PDGF (Asokananthan *et al.*, 2002; Shimizu *et al.*, 2000; Vliagoftis *et al.*, 2000). A separate study revealed that synthetic peptide agonists of both PAR-1 and PAR-2 stimulated the release of IL-6 and IL-8 from bronchial epithelial cell lines and primary NHBE cells, however, the PAR-2 peptide agonist was determined to be more effective than that of PAR-1 at 400 μM (Asokananthan *et al.*, 2002). The same order of efficacy was observed in the current study whereby the effect of PAR-2 activation on the levels of fibrinogen release from 16HBE 14o$^-$ cells was significantly *greater* than that of PAR-1 at baseline. The effect of PAR-1 activation on the release of fibrinogen, FXIII and D-dimers was proportional to the extent of wounding whereas activation of PAR-2 stimulated maximal responses at baseline. PAR-mediated cytokine release is controversial as Page *et al.* (2003) demonstrated that PAR-2 activation with 0.05 μM peptide agonist stimulated IL-8 release from 16HBE 14o$^-$ cells, whereas activation of PAR-1 did not. Others have shown that activation of both PAR-1 and PAR-2 stimulated the release of IL-8 when peptide agonists were used at 400 μM (Asokananthan *et al.*, 2002). Due to the high concentration of PAR peptide agonists that was employed in both the current study and

that of Asokananthan *et al.*, the possibility of ligand cross-reactivity may not be ruled out as Blackhart *et al.* demonstrated that specificity of the xenopus PAR-1 peptide agonist TFRIFD-amide was observed at concentrations up to 50 μM. Therefore, specificity of PAR activation may be reduced at higher concentrations.

PAR-2 activation is known to elicit a range of cytoprotective effects including wound healing, which is driven largely by release of the secondary mediator PGE_2 from the epithelium (Cocks *et al.*, 2000). Moreover, activation of PAR-1 in addition to PAR-2 has been shown to stimulate the release of PGE_2 from A549 cells and primary NHBE cells (Asokananthan *et al.*, 2002). However, PGE_2 was not involved in the current study as indomethacin was without effect. Nevertheless, a cytoprotective effect of PAR-1 and PAR-2 mediated *via* fibrin formation and enhanced epithelial repair is indicated.

PARs are believed to play an important role in coagulation and inflammation. However, a recurring observation is the ability of PAR-activators to mediate both pro- and anti-inflammatory effects. This chapter has demonstrated that activation of both PAR-1 and PAR-2 stimulates the release of coagulation factors, which are important in wound repair. However, over-stimulation of PARs could have detrimental effects such as excessive fibrin formation or over-production of inflammatory mediators. It must be noted that the current understanding of the relative contribution of PARs to pro-inflammatory and cytoprotective processes during airway disease is incomplete. Further investigations are required, ideally with the use of novel PAR agonists and antagonists in order to provide a clearer overview for the role of PARs in the airways during homeostasis and disease states, and a better understanding of airway PARs when assessing their potential as therapeutic targets.

Chapter 6.

The Effect of Wounding on: Coagulation Factor, Prostaglandin Receptor and PAR Expression at the mRNA Level

6. The effect of wounding on: coagulation factor, prostaglandin receptor and PAR expression at the mRNA level

6.1. Introduction

TF, the initiator of the coagulation cascade is normally expressed on cells extrinsic to the vascular compartment, however, endothelial cells, macrophages and monocytes can be induced to express TF by inflammatory mediators *via* a mechanism that commonly involves calcium and PKC (Camerer *et al.*, 1996; Pettersen *et al.*, 1992; Ternisien *et al.*, 1993; Yang *et al.*, 1994). Upregulation of TF expression in these cells is achieved by oxidised low density lipoprotein, thrombin, cytokines (IL-1, TNF-α) mitogens (FGF, PDGF, EGF, insulin, HGF, TGF-β1, VEGF), endotoxins, viral infections, IFN and occupancy of cell adhesion molecules (Camerer *et al.*, 1996) as well as FXa (Leadley *et al.*, 2001). LPS induces TF expression in both monocytes and endothelial cells *via* interactions with the LPS binding protein/CD14 complex (Read *et al.*, 1993; Steinemann *et al.*, 1994). TF expression and activity has also been demonstrated on primary NHBE cells in culture (Keller *et al.*, 2001).

The liver was once considered to be the sole source of FVII, whereby its production is regulated by the liver-enriched transcription factor hepatocyte nuclear factor-4 (HNF-4) as well as transcription factor SP-1 (Greenberg *et al.*, 1995). Since then, expression of FVII mRNA has been demonstrated in macrophages, smooth muscle cells, fibroblasts and keratinocytes (Wilcox *et al.*, 2003). However, expression of FVII in epithelial cells remains to be investigated.

The liver is the main source of FX and gene expression is regulated by the transcription factor HNF-4 (Hung *et al.*, 1996; Hung *et al.*, 2001). Low levels of FX are synthesised in extra-hepatic tissues including the lung (Hung *et al.*, 1996). The FX mRNA found in the lung may be from a number of cell types including the bronchial epithelium as well as alveolar macrophages, which are a source of the protein (Osterud *et al.*, 1980).

Fibrinogen is mainly derived from the liver and during inflammation the systemic levels of fibrinogen are enhanced by the actions of IL-6 and glucocorticoids (Herrick *et al.*, 1999). In addition to hepatic synthesis, transfection and expression of fibrinogen has been demonstrated in a COS cell line and yeast (Zhang *et al.*, 1996). Moreover, the A549 cell line derived from the alveolar basal epithelial cells has been demonstrated to express fibrinogen mRNA following stimulation with IL-6 and dexamethasone (Haidaris, 1997).

The major sites of production of FXIII subunit A (FXIIIA) are from cells originating from the bone marrow, including megakaryocytes, platelets, macrophages and monocytes, whereas the liver only plays a minor role in its synthesis (Adany *et al.*, 2003; Muszbek *et al.*, 1996). It has been demonstrated that *cis*-elements and *trans*-acting factors are involved in the constitutive and cell-type specific expression of FXIIIA. These include hematopoietic- or myeloid-specific elements such as MZF-1, GATA-1, and Ets-1 as well as general elements such as NF-1 and SP-1 (Ichinose, 2001; Kida *et al.*, 1999). Expression of FXIIIA mRNA has been demonstrated in homogenates of lung tissue, but the specific cell type from which it originated was not determined (Kaczmarek *et al.*, 1995). The liver is the sole site for the synthesis of the FXIII subunit B (FXIIIB) which forms a heterotetrameric complex with FXIIIA in plasma (Muszbek *et al.*, 1996).

It is established that within the respiratory tract, PAR-1 and PAR-2 are abundant in the bronchial epithelium (Knight *et al.*, 2001) as well as airway smooth muscle (ASM) cells, endothelial cells and vascular smooth muscle cells (Lan *et al.*, 2002). Tissue-resident macrophages have been demonstrated to express PAR-1 and PAR-2 (Knight *et al.*, 2001). PAR-1 and PAR-2 expression is also attributed to migratory cells such as mast cells and polymorphonuclear cells (Lan *et al.*, 2002). Studies of cultured primary NHBE cells and cell lines have confirmed that different cell types express different PAR profiles. This may reflect selective responses to a variety of proteases acting in the airways. Studies involving RT-PCR analysis have demonstrated the presence of both PAR-1 and PAR-2 mRNA in A549 and BEAS-2B cell lines as well as NHBE cells (Asokananthan *et al.*, 2002) and 16HBE 14o$^-$ cells (Page *et al.*, 2003). Moreover, PAR activation experiments have indicated that PAR-1 and PAR-2 are expressed by the 16HBE 14o$^-$ cell line under the conditions used in the current study (*Chapter 5*).

Regulation of PAR activity at the gene transcriptional level may be achieved by a variety of inflammatory mediators, which may induce or suppress both receptor gene expression and the presentation of the receptor protein on the cell surface. Regulation can also occur at the level of functional activity as a result of PAR activation/inactivation by endogenous or exogenous proteases (Sokolova *et al.*, 2007). *In vitro* studies have demonstrated several potential modulators of PAR expression. In endothelial cells, PAR-1 and PAR-2 gene expression was enhanced by macrophage migration inhibitory factor, a proinflammatory cytokine which participates in the inflammatory phase of the wound healing process (Shimizu *et al.*, 2004). The proinflammatory, asthma-associated cytokine IL-1β has been demonstrated to stimulate the expression of PAR-2 in human ASM cells (Freund-Michel *et al.*, 2006). In fibroblasts, PAR-2 expression may be stimulated by the profibrotic growth factors TGF-β1 and PDGF (Gruber *et al.*, 2004). More recently, the proinflammatory mediators LPS and TNF-α were demonstrated to enhance PAR-2 expression in fibroblasts (Ramachandran *et al.*, 2007). In macrophages, the asthma-associated cytokine IL-4 was demonstrated to downregulate PAR-1, PAR-2 and PAR-3 both at the mRNA and protein level (Colognato *et al.*, 2003). Interestingly, it is reported that persistent PAR activation can also influence PAR expression. For example, continuous activation of PAR-1 and PAR-2 by peptide agonists resulted in upregulation of these receptors in the A549 cell line (Sokolova *et al.*, 2007).

The receptors for PGE_2 have been classified into four subtypes: EP-1, EP-2, EP-3 and EP-4. The tissue distribution and intracellular signalling pathways used by the subclasses of EP receptors differ substantially (Narumiya *et al.*, 1999). These differences may explain the wide array of effects that are induced by PGE_2. According to Savla *et al.*, the regulation of wound repair by PGE_2 was mediated *via* EP-1 and EP-4. However, the stimulatory effects of PGE_2 were not reproduced in the current model.

Studies using RT-PCR analysis have detected mRNA corresponding to all four receptors in the mouse lung (Katsuyama *et al.*, 1995; Ushikubi *et al.*, 1995). EP receptor distribution among cells within the lung is not well established, however, it is reported that various airway cell types can express multiple EP receptor subtypes (Tilley *et al.*,

2003). EP receptors are reported to be regulated by proinflammatory stimuli *in vitro*. Expression of EP receptors is regulated by several factors through action on *cis*-acting regulatory elements of the respective receptor genes (Narumiya *et al.*, 1999). However, at present there is limited information available regarding this regulation. Stimulation with serum and bacterial LPS has been demonstrated to upregulate EP receptor expression on fibroblast and macrophage cell lines respectively. However, *in vivo* regulation of EP receptor expression has not yet been demonstrated.

6.2 Aims and objectives

To determine the expression level of mRNA for TF, FVII, FXa, fibrinogen, FXIII, PAR-1, PAR-2 and EP-1, EP-2, EP-3 and EP-4 in 16HBE 14o$^-$ cells at baseline and in response to wounding.

6.3. Methods

6.3.1. Total RNA isolation

16HBE 14o$^-$ cells were seeded at a density of 400,000 cells per well in 6-well plates and cultured for 48 hours in 2 ml per well of full MEM until fully confluent. At confluence, cells were quiesced for 16 hours in 1 ml per well of serum-free MEM-ITS. Prior to experiment, MEM-ITS was refreshed. Cells were maximally wounded, i.e. 4 horizontal scrapes and 4 vertical scrapes, as described in *section 2.2.5* and subsequently incubated for 2, 4, 6, 8 and 10 hours at 37°C. One 6-well plate was used for each time point. Following incubation, the cell culture supernatants were removed and harvested and cells were washed with 500 µl per well of 1X PBS then trypsinised by incubating with 250 µl per well of 1X trypsin-EDTA for 5 minutes at 37°C. Once the cells had lifted, 250 µl per well of FBS was added to neutralise trypsin activity. The cell suspension from each well of the 6-well plate was combined and removed into 15 ml centrifuge tubes and centrifuged for 7 minutes at 670 x *g* (ALCPK120 Centrifuge, Winchester Virginia, USA) to create a cell pellet. To each tube, 600 µl of pre-chilled denaturing solution (Promega) was added, after which the mixture was homogenised (ULTRA-

TURRAX T25, Janke & Kunkel IKA Labortechnik, Germany) at 24,000 revolutions per minute for 1 minute at room temperature. To the homogenate was added 60 µl of 2.0 M sodium acetate (pH 4.0) (Promega) and the contents of the tube were thoroughly mixed by inversion of the tube 5 times. To each tube, 600 µl of phenol:chloroform:isoamyl alcohol (99:24:1, pH 4.7) (Promega), drawn from the lower, organic phase of the mixture was added, and the tubes were mixed by inversion 5 times and vigorously shaken for 10 seconds. The tubes were then chilled on ice for 15 minutes. The contents of the tube were then transferred to clean, sterile 1.5 ml centrifuge tubes, and centrifuged at 10,000 x *g* for 20 minutes at 4°C. The top aqueous phase of the mixture containing RNA was carefully removed using a pipette without disturbing the protein interface and DNA lower phase. The RNA-containing upper phase was transferred to a sterile 1.5 ml centrifuge tube and mixed with an equal volume of isopropanol and incubated at -20°C for 30 minutes in order to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 x *g* for 20 minutes at 4°C, after which the supernatant was decanted and the pellet washed with 1 ml of ice-cold 75% ($^{v}/_{v}$) ethanol. The pellet was broken up using a 1 ml sterile, RNase-free pipette tip and the tubes were centrifuged at 10,000 x *g* for 20 minutes at 4°C. The pellets were then air-dried in an RNase-free environment for 15 minutes, dissolved in 30 µl of nuclease-free water after which RNA concentration and purity were estimated by optical density (OD) determination. The RNA concentration in solution was determined at 260 nm and 280 nm based on the formula that an absorbance of 1 in a 1 cm path quartz cuvette at 260 nm is approximately equivalent to 40 µg/ml. The 260/280 ratio was usually >1.6, indicating suitable purity of RNA.

6.3.2. Determination of RNA concentration by spectrophotometry

As described in *section 6.3.1*, the concentration of extracted RNA was determined by measurement of absorbance at 260 nm (A260) in a spectrophotometer (ANTHOS spectrophotometer, Cambridge Instruments, UK). The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb UV radiation, such as any proteins present in the solution. To ensure accuracy, valid readings were only accepted when the ratio was equal to or greater than 1.6. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per

ml. This relationship is valid only for measurements in deionised water. Therefore, all necessary dilutions of the RNA in solution were performed in sterile water. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. For measurement of RNA samples, an RNase-free quartz cuvette was used; this was accomplished by washing the cuvette with 0.1 M NaOH, 1 mM EDTA followed by washing with RNase-free water. A blank calibration reading was achieved using the buffer in which the RNA is diluted. This provided the zero value for subsequent spectrophotometric analysis of RNA samples.

Samples were then diluted accordingly in order to use 1 µg of total RNA for first strand synthesis.

6.3.3. Determination of RNA quality

In order to determine the quality of the isolated RNA, 2 µl of the extracted RNA solution was mixed with 3 µl of loading dye (10 mM Tris, 50 mM EDTA, 10% ($^{v}/_{v}$) glycerol, 0.25% ($^{w}/_{v}$) bromophenol blue, 0.25% ($^{w}/_{v}$) xylene cyanol FF, 0.4% ($^{w}/_{v}$) orange G) and electrophoresed at 150 V for 18 minutes on a 1% ($^{w}/_{v}$) agarose (BDH) gel containing 0.0001% ($^{v}/_{v}$) ethidium bromide until the dye front had travelled 75% along the total gel length.

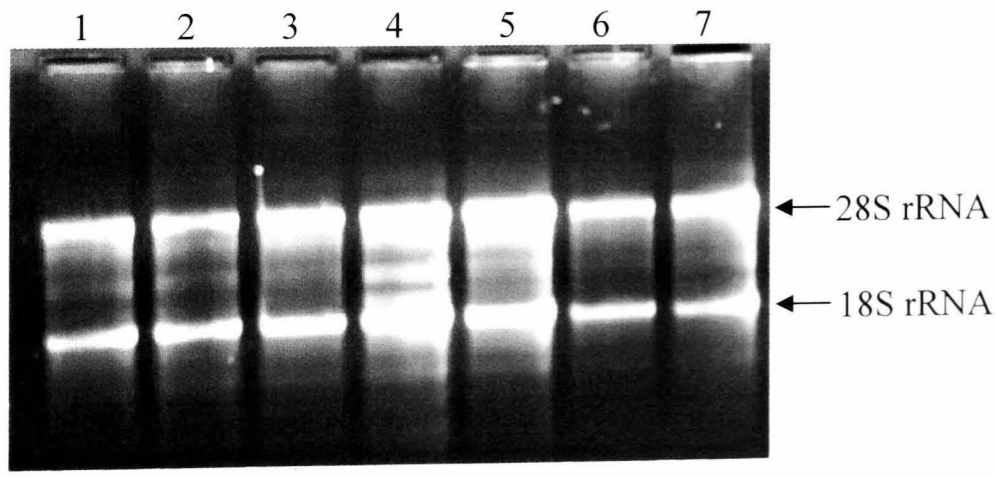

Figure 6.1. Representative gel demonstrating the presence of 28S rRNA and 18S rRNA in samples following RNA isolation.
Lane contents as follows: (1) Baseline (unwounded cells) (2) 2 hours *post*-wound (3) 4 hours *post*-wound (4) 6 hours *post*-wound (5) 8 hours *post*-wound (6) 10 hours *post*-wound (7) 12 hours *post*-wound.

6.3.4. Primer design

The cDNA sequence of the gene of interest was obtained using the Entrez Gene database available on the NCBI website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene). The NCBI Reference Sequences (RefSeq) was used to identify the RefSeq mRNA sequence of interest (both species- and enzyme-specific) with the corresponding accession number. The sequence link was then used to determine the actual sequence from the GenBank Nucleotide sequence database, which was subsequently pasted into the primer design program, 'Primer3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Forward and reverse primers were duly designed against a region of the gene in question according to the rules of primer design as outlined below:

Rules for primer design:

1. Primer sequences should be 17-28 bases in length;
2. Base composition should be 50-60% G and C ('GC rich');
3. Primers should end 3′ in a G or a C, or CG / GC. (i.e. 3′ clamp). This prevents 'breathing' of the ends and increases the efficiency of priming;
4. T_m (melting temperature) between 50-80 °C is the most preferable;
5. Runs of 3 or more Cs or Gs at the 3′ end of primers may promote mis-priming at G- or C-rich sequences (due to the stability of annealing) and should be avoided;
6. 3′-ends of primers should not be complementary, otherwise primer dimers may be synthesised preferentially to any other product;
7. Primer self-complementarity (ability to form secondary structures such as hairpins) should be avoided.

A potential problem with RT-PCR is the contamination of RNA with genomic DNA. Choosing primers that span introns is necessary in order to differentiate whether a band is amplified from genomic DNA or cDNA. If the primers span one or more introns and the RNA is contaminated with DNA, the result will be two bands, one of which will be from cDNA and the other from DNA. In order to determine whether the amplification is

from the cDNA template or from genomic DNA, RT-PCR primers are usually designed to span introns or to bridge an exon-exon junction.

Gene	Direction	Sequence	Product size (base pairs)	T_m (°C)
18SRNA	Forward	catgcatgtctaagtacgcacggcc	200	66.3
	Reverse	gggctgaccgggttggtttt		61.4
β-actin	Forward	gcgggaaatcgtgcgtgacatt	232	57.0
	Reverse	gatggagttgaaggtagtttcgtg		56.0
TF	Forward	caacagacacagagtgtgacctcaccgacg	502	66.0
	Reverse	tctctgaattccccttttctcctggccc		63.0
FVII	Forward	agctcatggtgctcaacgtg	404	53.5
	Reverse	atatgggatttggtgccagg		53.6
FGC	Forward	cttgaagcacagtgccagga	413	53.6
	Reverse	cccaccttgaacatggcata		53.1
FX	Forward	ttcgacctgcttgacttcaac	430	52.0
	Reverse	tcttctgcgtcatcagcgtg		54.7
FXIII	Forward	gaaacagacacgtacattct	445	48.0
	Reverse	aaaatgtgttaaagacacca		44.0
PAR-1	Forward	ggatatttgaccagctcctgg	400	54.0
	Reverse	agatggccagacaagtgaagg		54.0
PAR-2	Forward	ctgcatctgtcctcactggaa	400	54.0
	Reverse	attgccagggagatgccaatg		54.0
EP-1	Forward	gcgctgcccatcttctccatga	448	59.0
	Reverse	tgccccgggtactgcagctcata		59.0
EP-2	Forward	tctccttgttccacgtgctg	446	54.0
	Reverse	gagcaccgagacaatgagaa		52.0
EP-3	Forward	atgaaggagacccggggctacgga	515	63.0
	Reverse	cgcgtcttcatgtggctcgcatac		61.0
EP-4	Forward	gcgagtacagcaccttcatt	543	52.0
	Reverse	agatgaccatctggatctcg		52.0

Table 6.1. Forward and reverse primer sequences used for RT-PCR.

6.3.5. Internal control

6.3.5.1. 18S RNA and β-actin

18S RNA was chosen as a suitable internal control since it is well established to have a constant expression level that is independent of the treatment condition and shows less variance in expression across a variety of treatment conditions than β-actin and

GAPDH. However, because 18S rRNA is so abundant, it amplifies rapidly during RT-PCR, quickly exhausting the reaction reagents. It can therefore be difficult or even impossible to detect product from rare messages while remaining in the exponential phase of amplification for 18S rRNA. For this reason, β-actin was also used. β-actin encodes a ubiquitous cytoskeleton protein and is expressed at moderately abundant levels in most cell types (Kinoshita T *et al,* 1992). The level of expression has been shown to vary in some types; however, there is no evidence to suggest that expression levels vary in bronchial epithelial cell lines.

Expression of mRNA was analysed and presented as the ratio of the expression of the factor of interest to that of the internal control

6.3.6. First strand complementary DNA (cDNA) synthesis of TF

A 20 mM stock solution of dNTP (deoxynucleotide triphosphate) was prepared by adding equal volumes of dATP, dCTP, dGTP and dTTP (100 mM; Invitrogen), with the addition of nuclease-free water, each at a dilution of 1 in 5. The dNTP mix provides the units for which DNA molecules are constructed, with individual components each carrying a single nitrogenous base (adenine, cytosine, guanine and thymine). A 25 µl reaction mixture was prepared consisting of 15 µl of RNA, 1 µl of 20 mM dNTP, 1 µl of random hexamer (0.2 µg/µl; Invitrogen) and 8 µl of nuclease-free water in a 1.5 ml sterile tube. The reaction mixture was incubated at 70°C for 10 minutes in order to linearise the RNA, then the mixture was immediately chilled on ice. 8 µl of five times concentrated first strand buffer (Invitrogen), 4 µl of 0.1 M DTT (dithiothreitol; Invitrogen) and 1 µl of RNaseOUT inhibitor (40 U/µl; Invitrogen) were added to the reaction mixture before incubation for 10 minutes at room temperature. At this point a 10 µl aliquot of reaction mixture was removed and allocated for use as the negative control, prior to the addition of 2 µl of Superscript II Reverse Transcriptase (50 U/µl; Invitrogen), after which the reaction mixture followed by incubation at 42°C for 1 hour. Enzymatic activity was then removed by incubation a 70°C for 15 minutes, at which point the mixture was stored at -20°C until required.

6.3.7. RT-PCR for TF and 18S RNA

A single host mix of 50 µl was created, consisting of 25 µl of two times concentrated Mastermix (Qiagen), 1 µl of forward (0.1 µg/ml) and 1 µl of reverse (0.1 µg/ml) primers for either TF or 18S RNA (Sigma-Genosys Ltd), 1 µl of complementary DNA which acted as a template for RT-PCR and 22 µl of nuclease-free water. RT-PCR reactions were subjected to 10 minutes at 72°C to linearise the cDNA, then 30 cycles or 17 cycles of amplification for TF and 18S RNA respectively, consisting of denaturation at 94°C for 20 seconds, annealing at 57°C for 20 seconds and extension for 40 seconds at 72°C. This was followed by a final extension step of 2 minutes at 72°C.

6.3.8. One-step RT-PCR

The SuperScript™ One-Step RT-PCR system (Invitrogen) was subsequently adopted for its convenient, sensitive and reproducible detection of RNA. Both cDNA synthesis and PCR are performed in a single tube using gene-specific primers and specific target RNAs from total isolated RNA. The system consists of two major components: SuperScript™ II RT/platinum® *Taq* Hi Fi Mix (a blend of SuperScript™ II Reverse Transcriptase and Platinum *Taq* DNA Polymerase High Fidelity) and 2X reaction mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM $MgSO_4$).

6.3.8.1. One-step RT-PCR for FVII, β-actin, fibrinogen gamma chain (FGC), FX, FXIII, PAR-1, PAR-2, EP-1, EP-2, EP-3 and EP-4

A single host mix of 50 µl was created, consisting of 25 µl of two times concentrated reaction mix (Invitrogen), 1 µl of forward (0.1 µg/ml) and 1 µl of reverse (0.1 µg/ml) primers for either FVII, β-actin, FGC, FX, FXIII, PAR-1, PAR-2, EP-1, EP-2, EP-3 or EP-4 (Sigma-Genosys Ltd), 1 µl of SuperScript™ II RT/platinum® *Taq* Hi Fi Mix, 1 µl of template RNA and 21 µl of nuclease-free water. RT-PCR reactions were subjected to 50°C for 30 minutes to synthesise the cDNA then pre-denaturation for 2 minutes at 94°C; followed by either 40 cycles of amplification (FVII, FGC, FX, FXIII, PAR-1, PAR-2, EP-1, EP-2, EP-3 and EP-4) or 30 cycles of amplification (β-actin), consisting

of denaturation at 94°C for 15 seconds, annealing at either 55°C (FGC, FX and FXIII), 56°C (EP-2), 57°C (EP-4), 60°C (FVII, β-actin, PAR-1 and PAR-2), 64°C (EP-1) or 66°C (EP-3) for 20 seconds and extension for 45 seconds at 68°C. This was followed by a final extension step of 10 minutes at 72°C.

6.3.9. Analysis of RT-PCR products by agarose gel electrophoresis

10 µl of each RT-PCR product was mixed with 2 µl of loading dye (10 mM Tris, 50 mM EDTA, 10% ($^{v}/_{v}$) glycerol, 0.25% ($^{w}/_{v}$) bromophenolblue, 0.25% ($^{w}/_{v}$) xylenecyanol FF, 0.4% ($^{w}/_{v}$) orangeG) and run at 150 V for 18 minutes on a 2% ($^{w}/_{v}$) agarose (BDH) gel containing 0.0001% ($^{v}/_{v}$) ethidium bromide until the dye front had travelled 75% along the total gel length. A 100 bp DNA ladder (Invitrogen) was also loaded. Bands were visualized by ethidium bromide fluorescence under ultraviolet light. Densitometry was then carried out using SynGene software; to semi-quantify the relative abundance of mRNA bands encoding each gene sequence compared with the internal control 18S RNA or β-actin.

6.4. Results

6.4.1. TF

6.4.1.1. Optimisation of conditions for RT-PCR amplification of TF

In order to optimise RT-PCR conditions for TF, the effect of cell cycle number on the abundance of the TF amplicon was investigated.

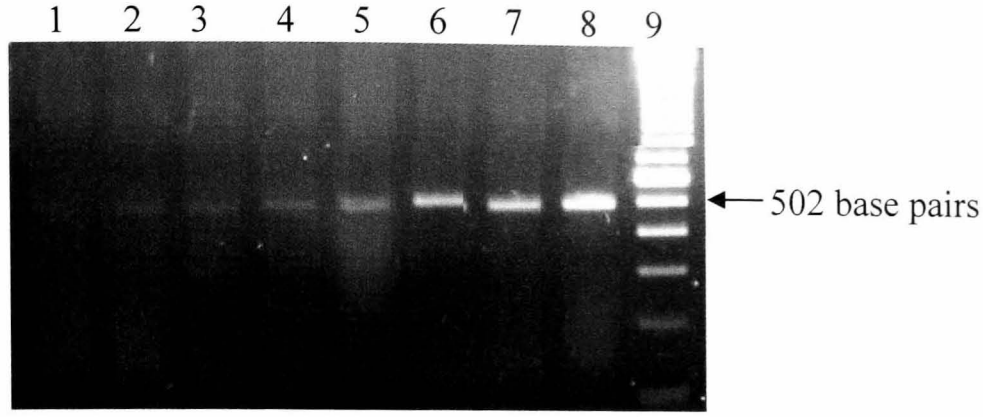

Figure 6.2. Representative gel demonstrating the effect of cell cycle number on mRNA expression of TF in 16HBE 14o$^-$ cells (23-30 cycles of amplification).
2% ($^w/_v$) agarose gel electrophoretic separation of TF amplicons generated by RT-PCR using cDNA synthesised from total RNA extracted from 16HBE 14o$^-$ cells at baseline. Lane contents as follows: (1) 23 cycles (2) 24 cycles (3) 25 cycles (4) 26 cycles (5) 27 cycles (6) 28 cycles (7) 29 cycles (8) 30 cycles (9) 100 base pair ladder.

With reference to *figure 6.2,* it was evident that linearity for TF mRNA with cycle number was achieved up to 30 cycles of amplification. Higher cycle numbers lead to saturation of the TF amplicon, therefore, 30 cycles of amplification were chosen for the RT-PCR conditions.

6.4.1.2. 18S RNA internal control

In order to optimise RT-PCR conditions for the internal control 18SRNA, the effect of cell cycle number on the abundance of the 18SRNA amplicon was investigated.

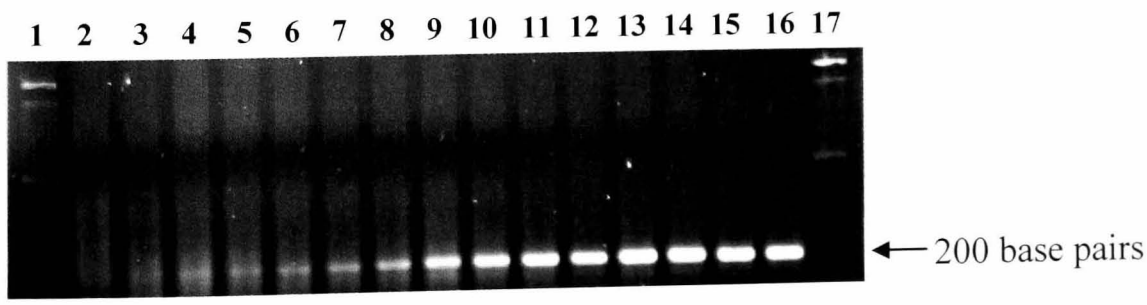

Figure 6.3. Representative gel demonstrating the effect of cell cycle number on mRNA expression of 18S RNA in 16HBE 14o$^-$ cells (10-24 cycles of amplification).
Lane contents as follows: (1) 100 base pair ladder (2) 10 cycles (3) 11 cycles (4) 12 cycles (5) 13 cycles (6) 14 cycles (7) 15 cycles (8) 16 cycles (9) 17 cycles (10) 18 cycles (11) 19 cycles (12) 20 cycles (13) 21 cycles (14) 22 cycles (15) 23 cycles (16) 24 cycles (17) 100 base pair ladder.

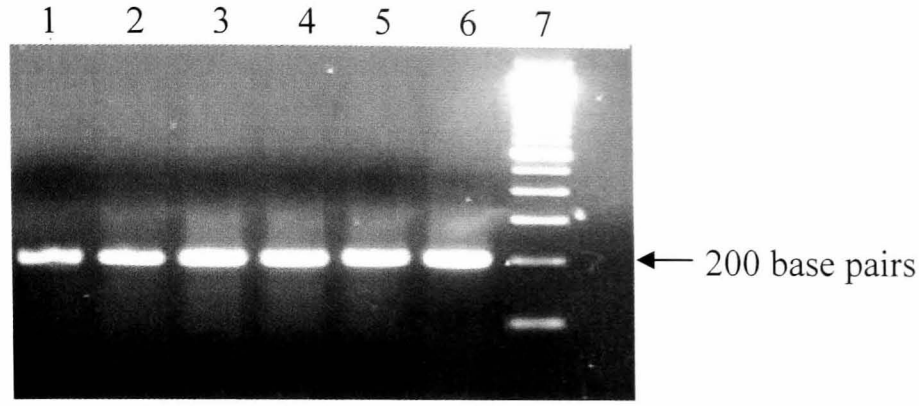

Figure 6.4. Representative gel demonstrating the effect of cell cycle number on 18S RNA mRNA expression in 16HBE 14o$^-$ cells (25-30 cycles of amplification).
Lane contents as follows: (1) 25 cycles (2) 26 cycles (3) 27 cycles (4) 28 cycles (5) 29 cycles (6) 30 cycles (7) 100 base pair ladder.

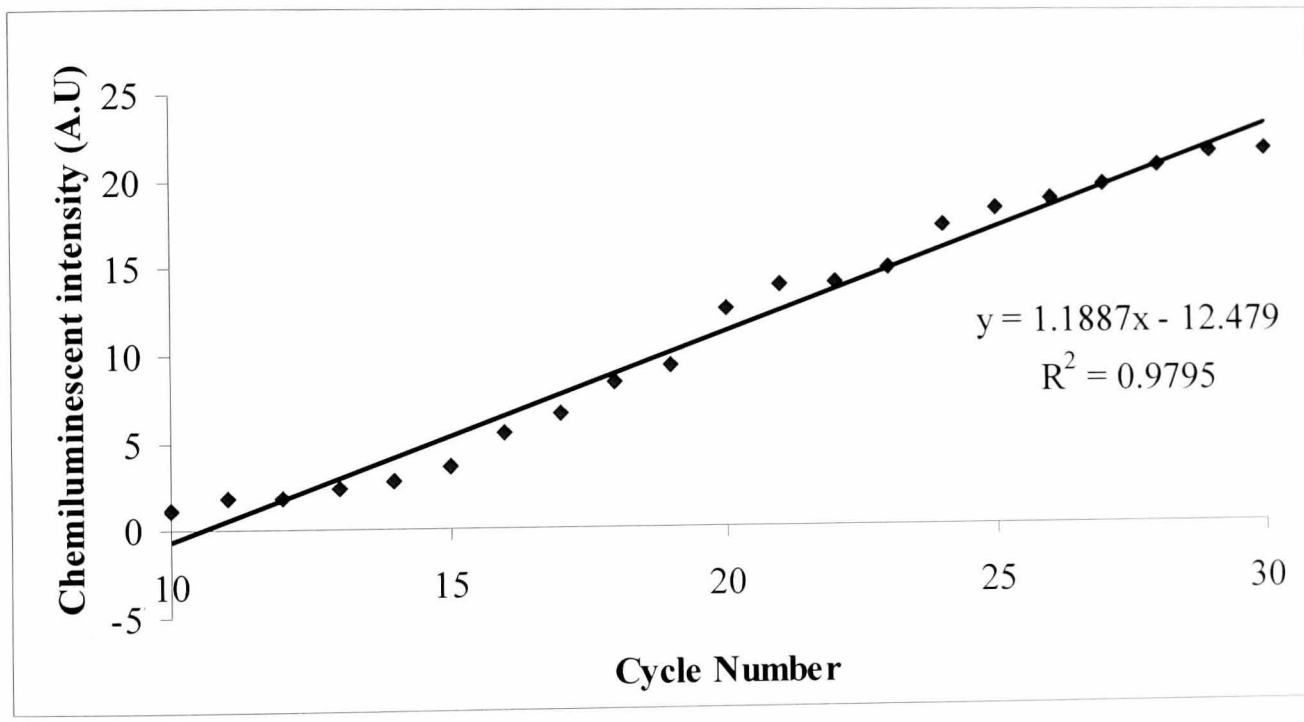

Figure 6.5. Determination of linearity for 18SRNA.

With regards to *figures 6.3* and *6.5*, it was decided that 17 cycles of amplification were to be used for future RT-PCR conditions as this was the point at which the abundance of the 18SRNA amplicon was comparable with that of TF, was within the linear range and had not yet become saturated.

6.4.1.3. TF mRNA expression *pre-* and *post-* wounding

The effect of wounding on mRNA expression of TF in 16HBE 14o$^-$ cells was analysed over 12 hours.

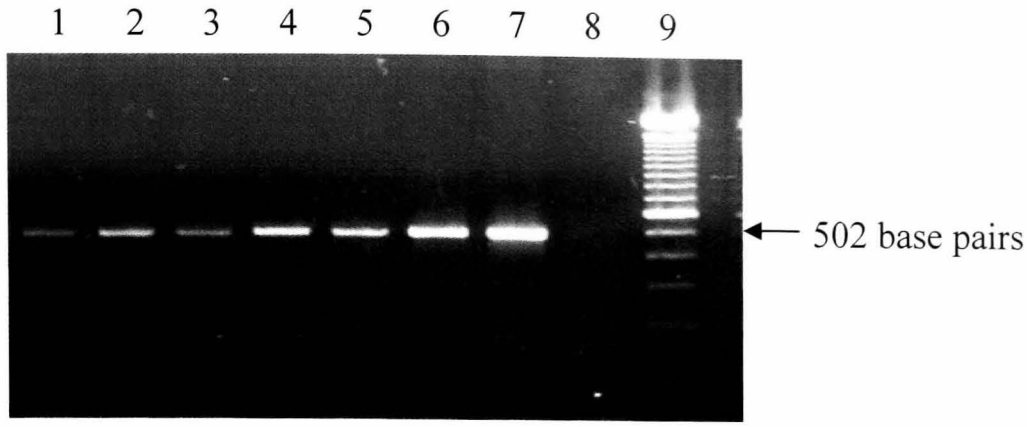

Figure 6.6. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of TF in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) Baseline (unwounded cells) (2) 2 hours *post*-wound (3) 4 hours *post*-wound (4) 6 hours *post*-wound (5) 8 hours *post*-wound (6) 10 hours *post*-wound (7) 12 hours *post*-wound (8) Negative control amplification using cDNA template prior to reverse transcription (9) 100 base pair ladder.

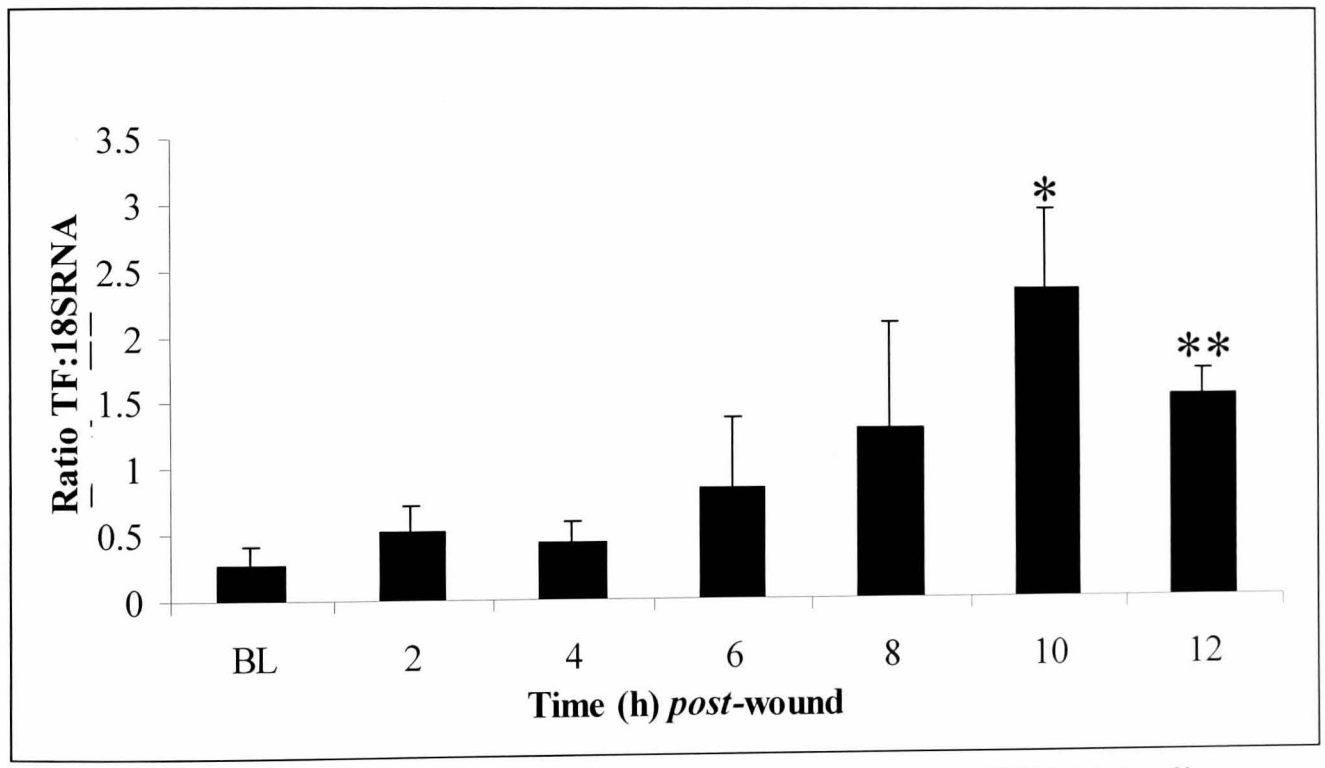

Figure 6.7. Effect of wounding on levels of mRNA for TF in 16HBE 14o$^-$ cells.
Data bars represent the ratio of TF to the internal control 18SRNA. Data represent mean ± SEM ($n=4$). BL=baseline i.e. unwounded cells. * Indicates $P<0.05$ and ** indicates $P<0.005$ compared to BL.

Immunohistochemical staining for TF in 16HBE 14o$^-$ cells demonstrated constitutive expression of this coagulation factor (*section 3.4.1*). RT-PCR analysis revealed that there was an increase in mRNA levels of TF that was time-dependent *post*-wounding. There was a significant increase in mRNA levels of TF at 10 hours (2.34 ± 0.61) and 12 hours (1.54 ± 0.20) *post*-wounding compared to baseline (0.27 ± 0.14).

6.4.2. FVII

6.4.2.1. β-actin internal control

The SuperScript™ One-Step RT-PCR system (Invitrogen) was subsequently adopted for its convenient, sensitive and reproducible detection of RNA and β-actin was used as the internal control.

The effect of wounding on mRNA expression of β-actin in 16HBE 14o$^-$ cells was analysed over 10 hours.

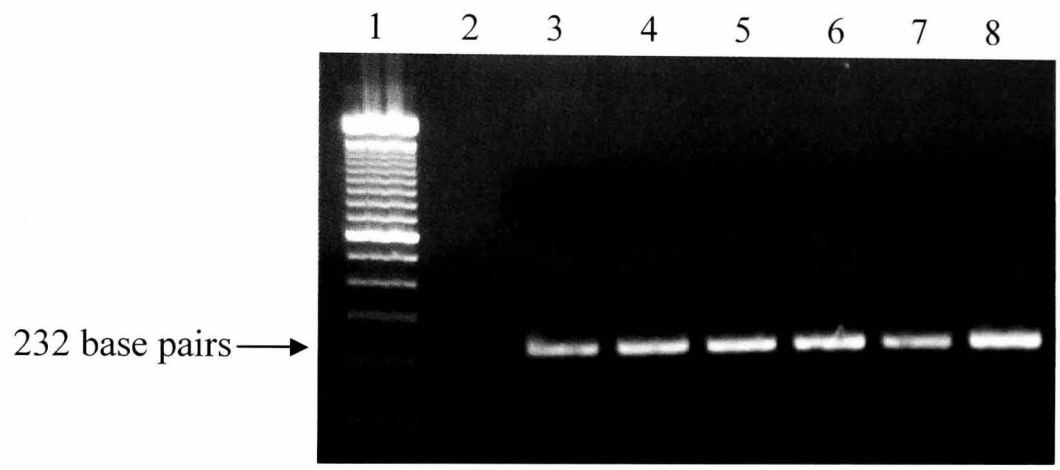

Figure 6.8. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of β-actin in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (no RNA) (3) Baseline (unwounded cells) (4) 2 hours *post*-wound (5) 4 hours *post*-wound (6) 6 hours *post*-wound (7) 8 hours *post*-wound (8) 10 hours *post*-wound.

Figure 6.8 indicates that β-actin mRNA is detected in 16HBE 14o$^-$ cells and that its expression does not change with time *post*-wounding, therefore β-actin was considered a suitable internal control for RT-PCR.

The effect of wounding on mRNA expression of FVII in 16HBE 14o$^-$ cells was analysed over 10 hours (*figure 6.9*).

6.4.2.2. FVII

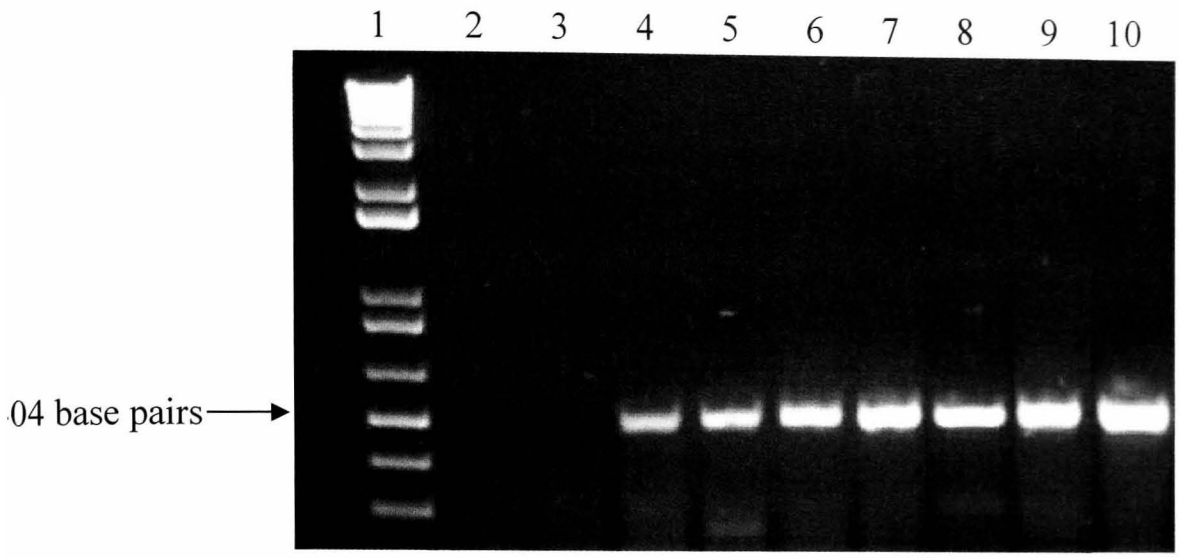

Figure 6.9. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of FVII in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (no RNA) (3) Negative control (no DNA polymerase enzyme) (4) Baseline (unwounded cells) (5) 2 hours *post*-wound (6) 4 hours *post*-wound (7) 6 hours *post*-wound (8) 8 hours *post*-wound (9) 10 hours *post*-wound (10) Liver RNA (positive control).

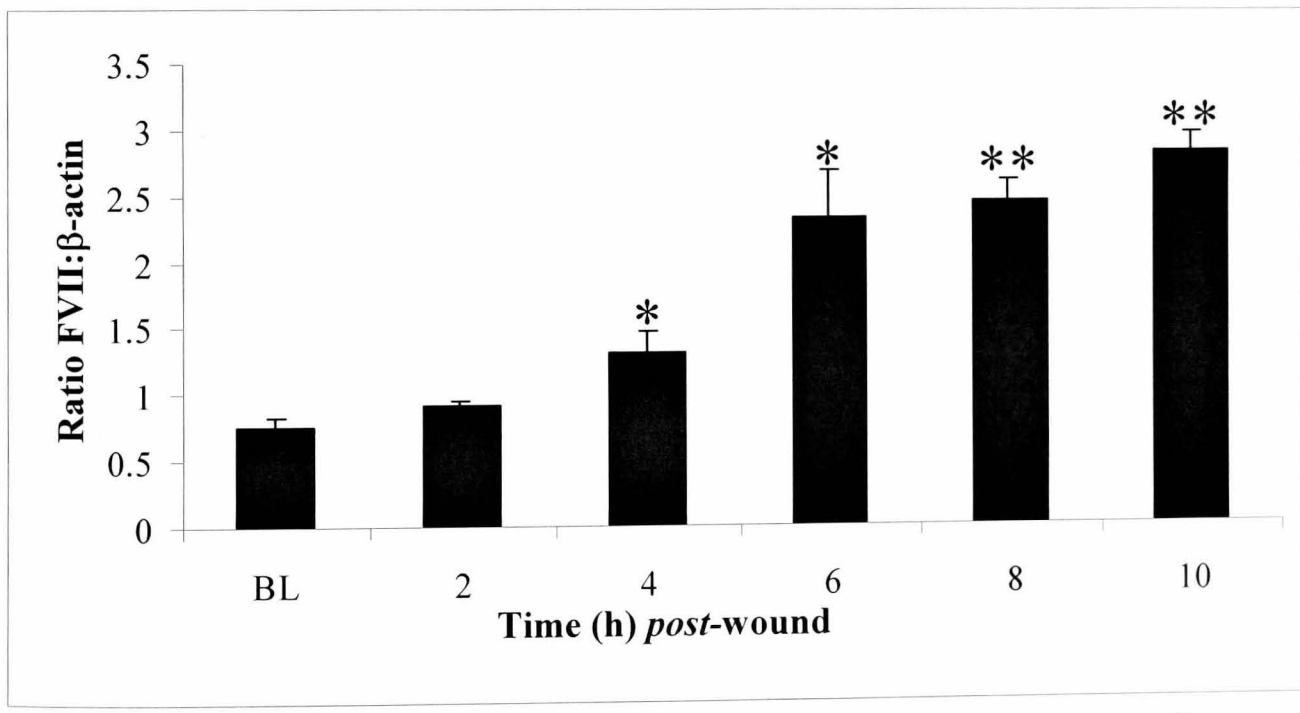

Figure 6.10. Effect of wounding on mRNA levels of FVII in 16HBE 14o$^-$ cells.
Data bars indicate the ratio of FVII to β-actin internal control. BL=baseline i.e. unwounded cells. Data represent mean ± SEM (*n=3*). * Indicates $P<0.05$ and ** indicates $P<0.0005$ compared to BL.

RT-PCR amplification demonstrated that FVII mRNA is detected in 16HBE 14o$^-$ cells and that there was a time-dependent increase in the level of FVII mRNA expression *post*-wounding. At 4 hours, mRNA expression levels were significantly higher (1.30 ±

0.03) compared to the unwounded cell cultures (0.76 ± 0.06). This effect was maintained up to 10 hours (6 hours = 2.33 ± 0.37; 8 hours = 2.47 ± 0.14 and 10 hours = 2.84 ± 0.14).

6.4.3. FGC and FXa

The effect of wounding on mRNA expression of FGC and FXa in 16HBE 14o$^-$ cells was analysed over 10 hours.

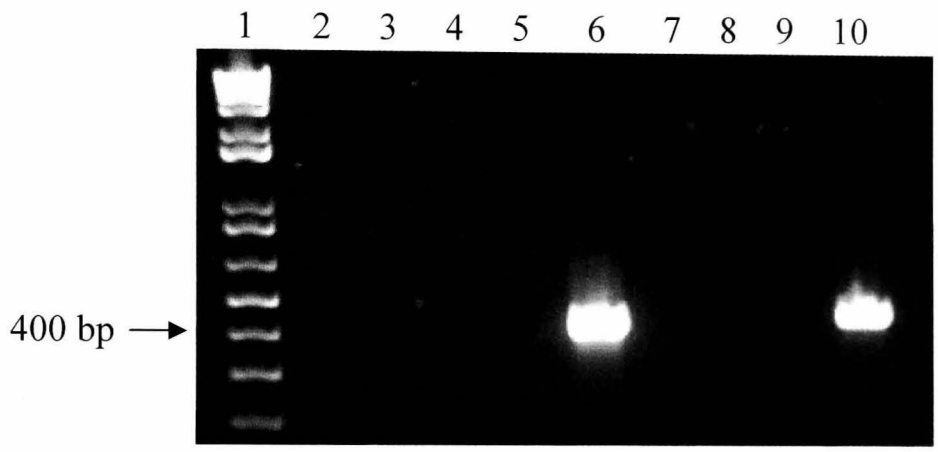

Figure 6.11. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of FGC and FXa in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) negative control (no RNA) (3) FGC: Baseline (unwounded cells) (4) FGC: 2 hours *post*-wound (5) FGC: 10 hours *post*-wound (6) FGC: liver RNA (positive control) (7) FXa: Baseline (unwounded cells) (8) FXa: 2 hours *post*-wound (9) FXa: 10 hours *post*-wound. (10) FXa: liver RNA (positive control).

Although fibrinogen protein expression in culture supernatants of 16HBE 14o$^-$ cells was determined by immunoblot (*section 3.4.2.1*), fibrinogen mRNA was not detected by RT-PCR in the same cell type. The fibrinogen primer pair was deemed valid since it was able to generate an abundant amplicon of 413 base pairs by RT-PCR, using RNA isolated from mouse liver (*figure 6.11*). Furthermore, in the absence of RNA, no amplicon was generated by RT-PCR.

Despite evidence that FXa is present in 16HBE 14o$^-$ cells due to inhibition of wound repair by two separate selective FXa inhibitors (*section 4.4.8.2*); FXa mRNA was not detected by RT-PCR in the same cell type. The FXa primer pair was deemed valid since it was able to generate an abundant amplicon of 430 base pairs by RT-PCR, using RNA

isolated from mouse liver (*figure 6.11*). Furthermore, in the absence of RNA, no amplicon was generated by RT-PCR.

In view of the evidence that both fibrinogen and FXa are present in 16HBE 14o$^-$ cell cultures, the possibility of gene repression by histone deacetylation was examined and preliminary results for fibrinogen and FXa are presented in *Chapter 7*, *sections 7.2.3.2* and *7.2.3.3* respectively.

6.4.4. FXIII

The effect of wounding on mRNA expression of FXIII in 16HBE 14o$^-$ cells was analysed over 10 hours.

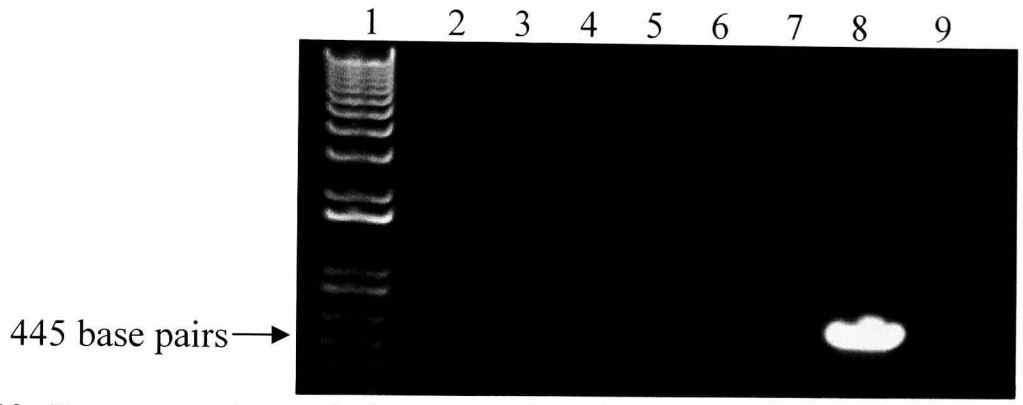

Figure 6.12. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of FXIII in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Baseline (unwounded cells) (3) 2 hours *post*-wound (4) 4 hours *post*-wound (5) 6 hours *post*-wound (6) 8 hours *post*-wound (7) 10 hours *post*-wound (8) liver RNA (positive control) (9) Negative control (no RNA).

Although FXIII protein expression in 16HBE 14o$^-$ cells was determined by immunoblot (*section 3.4.2.2*), FXIII mRNA was not detected by RT-PCR in the same cell type. The FXIII primer pair was deemed valid since it was able to generate an abundant amplicon of 445 base pairs by RT-PCR, using RNA isolated from mouse liver (*figure 6.12*). Furthermore, in the absence of RNA, no amplicon was generated by RT-PCR.

In view of the evidence that FXIII is present in 16HBE 14o$^-$ cell cultures, the possibility of gene repression by histone deacetylation was examined and preliminary results for FXIII are presented in *section 7.2.3.4*.

6.4.5. PAR-1

PARs 1-4 have previously been detected in NHBE cells and A549, BEAS-2B and 16HBE 14o$^-$ cell lines (Asokananthan *et al.*, 2002; Page *et al.*, 2003). Since 16HBE 14o$^-$ cells responded to PAR-1 and PAR-2 peptide agonists (*Chapter 5*), it was of interest to confirm the expression of PAR-1 and PAR-2 in 16HBE 14o$^-$ cells in the current study.

Figure 6.13 represents the effect of wounding on mRNA expression of PAR-1 in 16HBE 14o$^-$ cells over 10 hours.

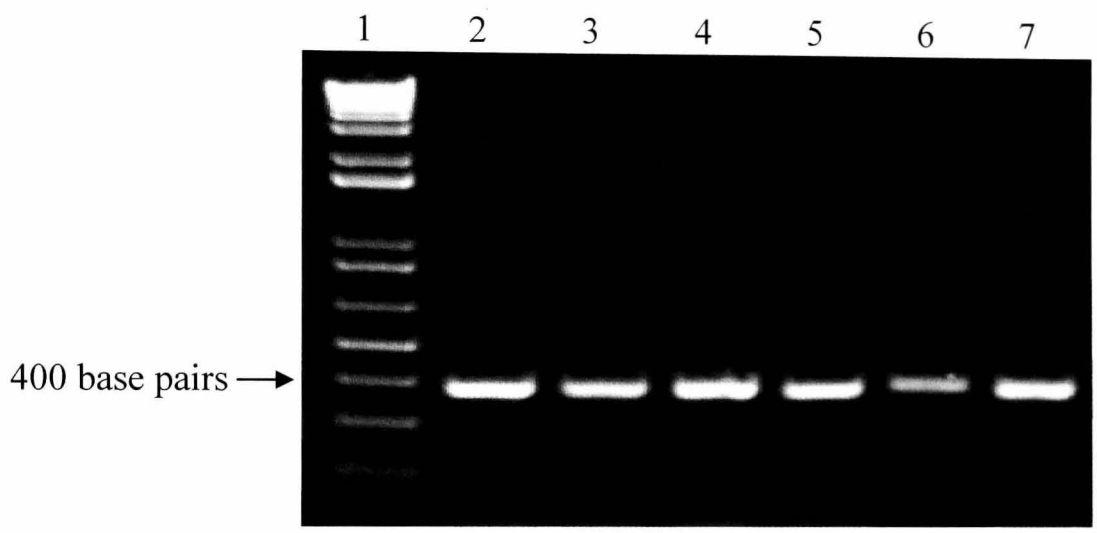

Figure 6.13. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of PAR-1 in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Baseline (unwounded cells) (3) 2 hours *post*-wound (4) 4 hours *post*-wound (5) 6 hours *post*-wound (6) 8 hours *post*-wound (7) 10 hours *post*-wound.

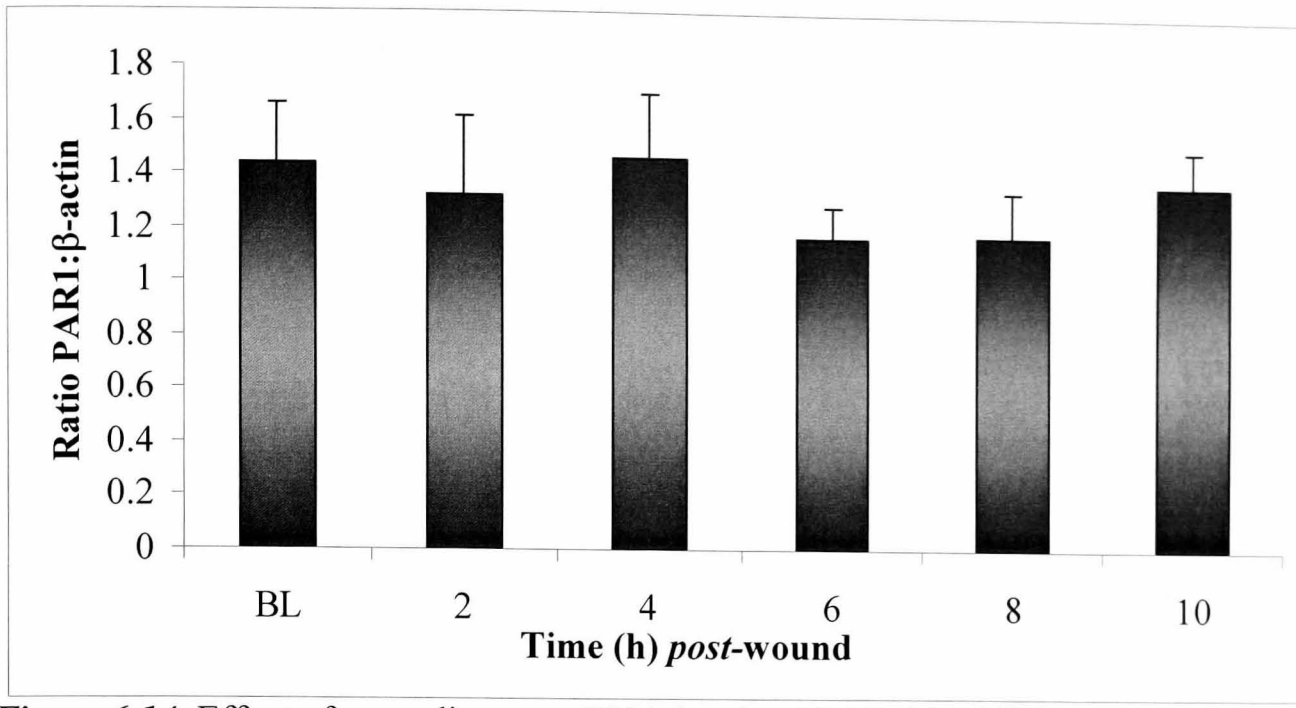

Figure 6.14. Effect of wounding on mRNA levels of PAR-1 in 16HBE 14o$^-$ cells. Data bars indicate the ratio of PAR-1 to β-actin internal control. BL=baseline i.e. unwounded cells. Data represent mean ± SEM (*n=3*).

RT-PCR amplification demonstrated that the PAR-1 receptor mRNA is detected in 16HBE 14o$^-$ cells; however, there was no change in PAR-1 mRNA expression with time *post*-wounding with regards to any of the time-points investigated or compared to baseline.

6.4.6. PAR-2

Figure 6.15 represents the effect of wounding on mRNA expression of PAR-2 in 16HBE 14o$^-$ cells over 10 hours.

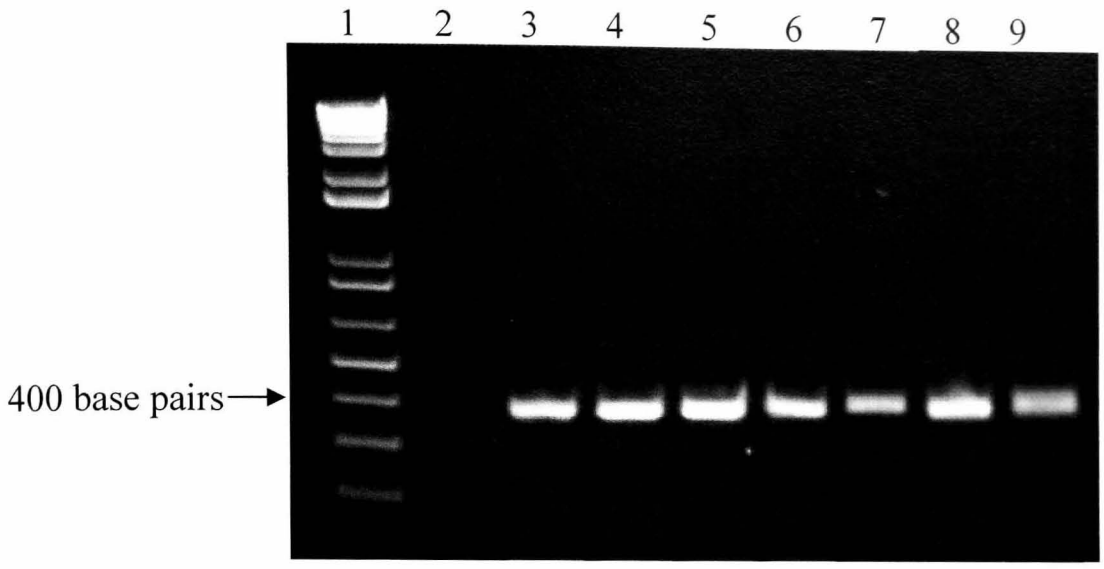

Figure 6.15. Representative gel illustrating the effect of time *post*-wounding on mRNA expression of PAR-2 in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (no RNA) (3) Baseline (unwounded cells) (4) 2 hours *post*-wound (5) 4 hours *post*-wound (6) 6 hours *post*-wound (7) 8 hours *post*-wound (8) 10 hours *post*-wound (9) Platelet RNA (positive control).

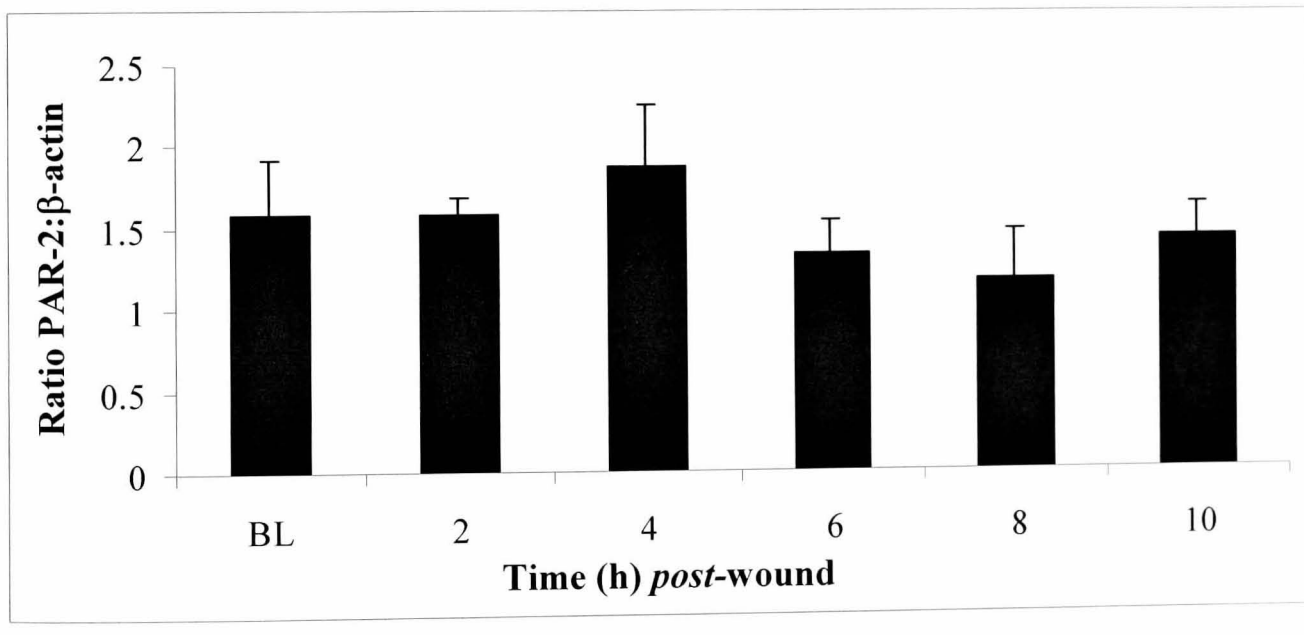

Figure 6.16. Effect of wounding on mRNA levels of PAR-2 in 16HBE 14o$^-$ cells.
Data bars indicate the ratio of PAR-2 to β-actin internal control. BL=baseline i.e. unwounded cells. Data represent mean ± SEM (*n=3*).

RT-PCR amplification demonstrated that PAR-2 is detected in 16HBE 14o$^-$ cells, however there was no change in PAR-2 mRNA expression with time *post*-wounding with regards to any of the time-points investigated or compared to baseline.

6.4.7. EP receptors

PGE_2 was previously reported to enhance bronchial epithelial repair in 16HBE $14o^-$ cells (Savla *et al.*, 2001). However, this effect was not reproduced in this study (*section 4.4.10.2*). However, when endogenous FXa was inhibited, there was evidence that prostanoids play a role in wound repair (*section 4.4.10.1*). Savla *et al.* reported that PGE_2 mediated wound repair *via* EP-1 and EP-4; therefore, it was of interest to investigate the presence of these receptors in the current model. For completeness, the presence of EP-2 and EP-3 were also investigated.

RT-PCR amplification demonstrated that mRNA for neither EP-1 nor EP-4 was detected in 16HBE $14o^-$ cells at baseline or in response to wounding. However, mRNA expression for both EP-2 and EP-3 was detected at baseline, thus, the effect of wounding on mRNA expression for these receptors was investigated.

Figure 6.17 represents the effect of wounding on mRNA expression of EP-2, the PGE_2 receptor in 16HBE $14o^-$ cells over 10 hours.

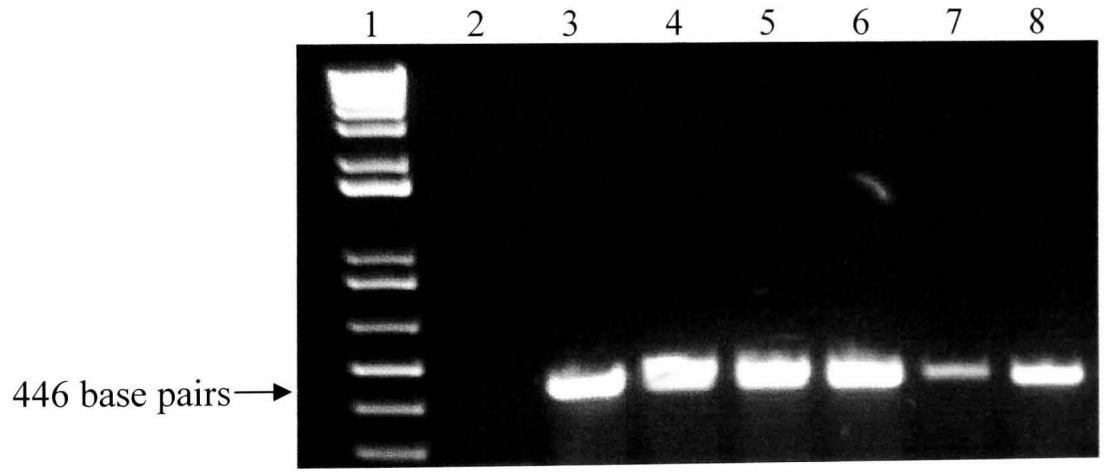

Figure 6.17. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of EP-2 in 16HBE $14o^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (no RNA) (3) Baseline (unwounded cells) (4) 2 hours *post*-wound (5) 4 hours *post*-wound (6) 6 hours *post*-wound (7) 8 hours *post*-wound (8) 10 hours *post*-wound.

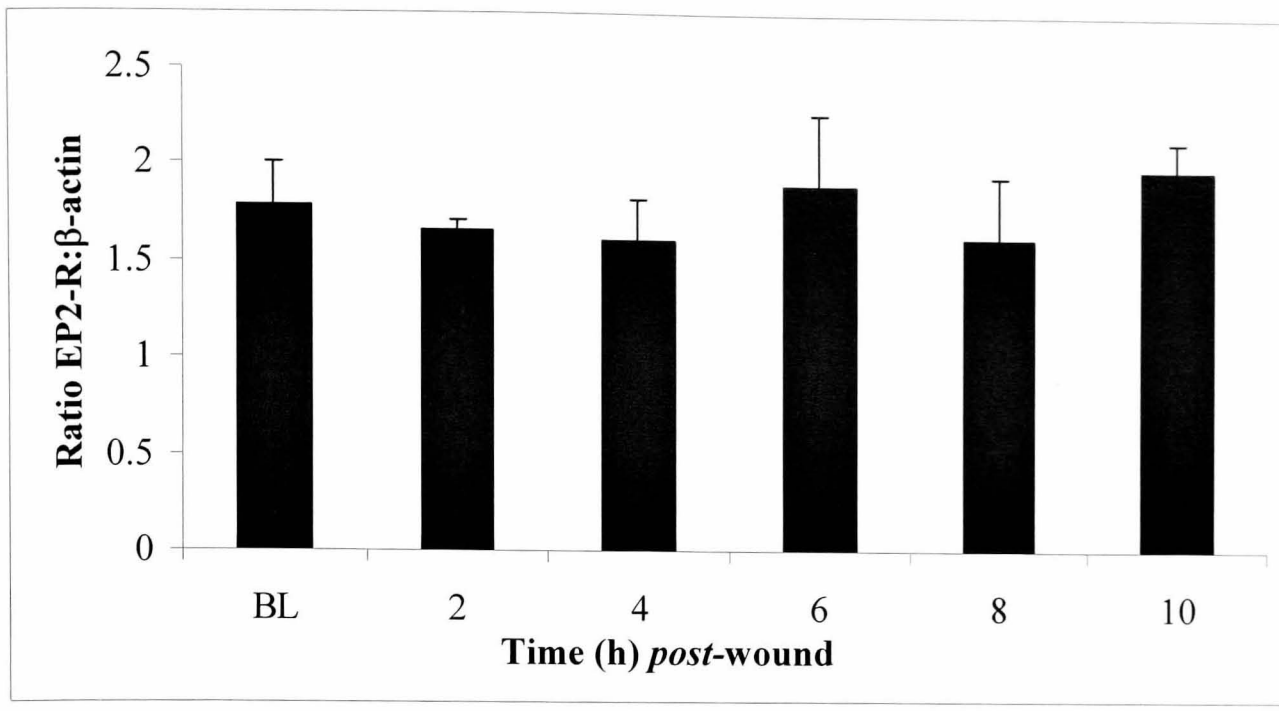

Figure 6.18. Effect of wounding on mRNA levels of EP-2 in 16HBE 14o$^-$ cells. Data bars indicate the ratio of EP-2 to β-actin internal control. BL=baseline i.e. unwounded cells. Data represent mean ± SEM (*n=3*).

RT-PCR amplification demonstrated that EP-2 mRNA is detected in 16HBE 14o$^-$ cells, however there was no change in EP-2 mRNA expression with time *post*-wounding with regards to any of the time-points investigated or compared to baseline.

Figure 6.19 represents the effect of wounding on mRNA expression of EP-3, the PGE_2 receptor in 16HBE 14o$^-$ cells over 10 hours.

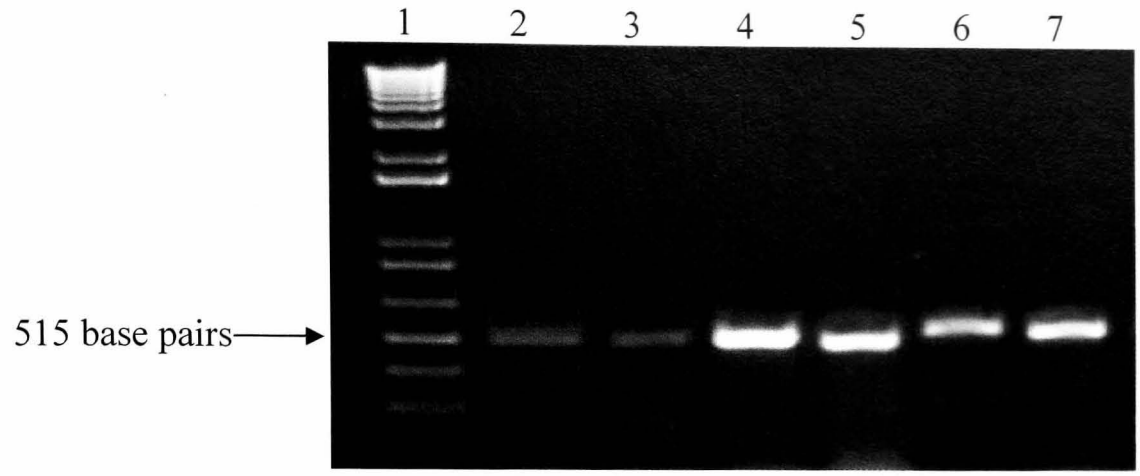

Figure 6.19. Representative gel demonstrating the effect of time *post*-wounding on mRNA expression of EP-3 receptor in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Baseline (unwounded cells) (3) 2 hours *post*-wound (4) 4 hours *post*-wound (5) 6 hours *post*-wound (6) 8 hours *post*-wound (7) 10 hours *post*-wound.

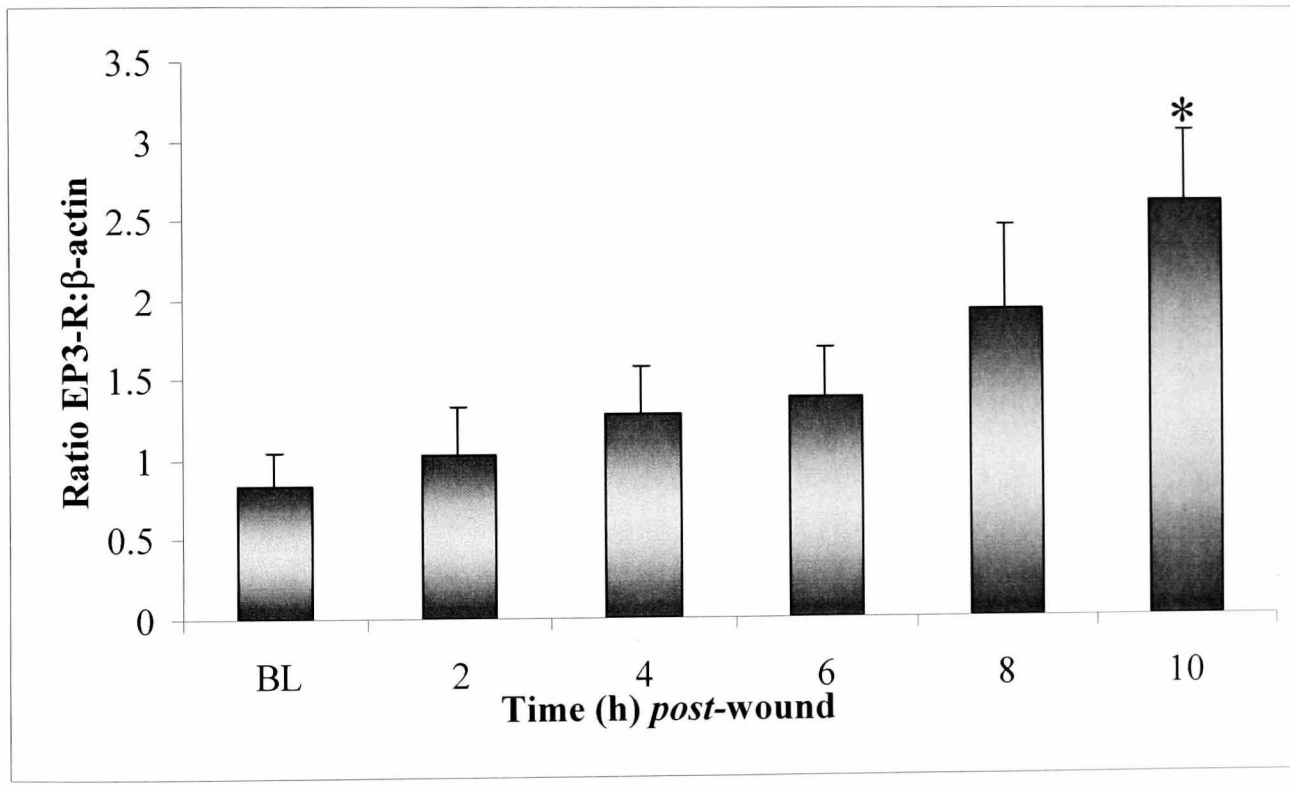

Figure 6.20. Effect of wounding on mRNA levels of EP-3 in 16HBE 14o$^-$ cells.
Data bars indicate the ratio of EP-3 to β-actin internal control. BL=baseline i.e. unwounded cells. Data represent mean ± SEM (*n=4*). * Indicates $P<0.05$ compared to BL.

RT-PCR amplification demonstrated that EP-3 mRNA is detected in 16HBE 14o$^-$ cells and that there was an increase in mRNA expression of EP-3 with time *post*-wounding (2 hours = 1.02 ± 0.30; 4 hours = 1.28 ± 0.30; 6 hours = 1.38 ± 0.32 and 8 hours = 1.93 ± 0.54) compared to baseline (0.84 ± 0.21) and at 10 hours (2.62 ± 0.46), the increase in expression was significant compared to baseline.

6.5. Summary of results

Both TF and FVII mRNA transcripts were detected at baseline in 16HBE 14o$^-$ cells and an increase in mRNA expression for each factor was demonstrated in a time-dependent manner *post*-wounding. Levels of significance of TF and FVII mRNA expression were achieved at 10 hours and 4 hours *post*-wound respectively, compared to baseline. Transcripts for FXa, fibrinogen and FXIII were not detected at baseline or in response to wounding of 16HBE 14o$^-$ cells. Both PAR-1 and PAR-2 mRNA was detected at baseline in 16HBE 14o$^-$ cells; however, there was no time-dependent change in mRNA expression *post*-wounding. Neither EP-1 nor EP-4 mRNA was detected at baseline in 16HBE 14o$^-$ cells or in response to wounding. EP-2 mRNA was detected at baseline in 16HBE 14o$^-$ cells and in response to wounding; however, there was no time-dependent change in mRNA expression *post*-wounding. Finally, EP-3 mRNA was detected at baseline in 16HBE 14o$^-$ cells and an increase in mRNA expression was demonstrated in a time-dependent manner *post*-wounding. A significant increase in EP-3 mRNA expression was achieved at 10 hours *post*-wound compared to baseline.

6.6. Discussion

The coagulation cascade depends on the formation of an initiation complex between TF and FVII. Thus, the expression of these coagulation factors at the mRNA level was investigated over the time-course of wound repair. RT-PCR amplification demonstrated the novel observation that 16HBE 14o$^-$ cells express TF mRNA. In accordance with immunohistochemical staining for TF, mRNA expression was detected in unwounded, confluent cell monolayers. Expression of TF mRNA increased in a time-dependent manner and levels of significance were achieved at 10 hours *post*-wounding.

Similarly, human platelets have recently been shown to synthesise their own TF for thrombin generation (Panes *et al.*, 2007). It was demonstrated that platelets contain TF mRNA, which is translated into *de novo* synthesised protein following activation. Expression of TF mRNA was observed following activation of platelets with a PAR-1 peptide agonist. Circulating platelets are reported to contain sufficient TF to account for the initiation of their clotting function and it has been suggested that TF is pre-formed

and rapidly exposed in the active form (Camera *et al.*, 2003; Muller *et al.*, 2003) or is more slowly decrypted (Bach, 2006) upon stimulation. In a separate study, platelet-monocyte complexes demonstrated mRNA expression of TF within 15 mins of PAR-1 activation and 2 hours *post*-stimulation with LPS (Lindmark *et al.*, 2000). Platelets are thus described as 'TF-bearing cells' as they enable the localisation of TF when required and are therefore self-sufficient in the initiation of fibrin formation.

Transcripts for FVII mRNA were also detected in unperturbed confluent 16HBE 14o$^-$ cells. Expression of FVII mRNA increased in a time-dependent manner and a significant increase was demonstrated 4 hours *post*-wounding. Expression of FVII mRNA by 16HBE 14o$^-$ cells is a novel observation. Since a significant increase in the release of FVII occurred rapidly, within 20 minutes *post*-wounding and FVII mRNA was not significantly increased until 4 hours *post*-wounding, it is evident that gene transcription is not responsible for FVII release in response to wounding. Instead, it is likely that FVII is preformed and stored within epithelial cells in order to be rapidly released following cell injury.

In hindsight, the design of the cell experiments to generate RNA for RT-PCR analysis was flawed as they displayed a lack of time-matched controls. If these experiments were to be repeated, they would include both unwounded and wounded cell cultures for each time-point investigated.

Expression of PAR-1 and PAR-2 mRNA in 16HBE 14o$^-$ cells was unaffected by wounding. However, an increase in the release of a PAR-activating protease such as HAT (PAR-2) or thrombin (PAR-1) could lead to upregulation of TF and possibly FVII expression. Thus, it would be interesting to investigate the expression of coagulation factors in response to PAR activation by incubating 16HBE 14o$^-$ cells with PAR agonists prior to RNA extraction.

Despite demonstration of FXa inhibition by two selective FXa inhibitors, determination of fibrinogen and FXIIIA protein in cell culture supernatants; inhibition of wound repair by anti-fibrinogen and anti-FXIIIA and immunostaining for FXIIIA, mRNA for FXa, fibrinogen and FXIIIA was not detected in 16HBE 14o$^-$ cells. The primer sets were deemed valid since each of them were able to generate an abundant amplicon of the

appropriate base pairs using RNA isolated from mouse liver. The absence of mRNA for these coagulation factors was illogical, therefore the possibility of gene repression was explored (see *Chapter 7*).

Expression of PAR-1 and PAR-2 was confirmed at the level of transcription in 16HBE 14o$^-$ cells in the current model. Expression of PAR-1 and PAR-2 mRNA by wounded monolayers did not change with time *post*-wounding compared to unwounded cells. Thus mechanical wounding had no influence on PAR expression.

Both exogenous and endogenous PGE_2 were previously reported to enhance wound repair of 16HBE 14o$^-$ cells (Savla *et al.*, 2001). However, these observations were not confirmed in the current model since both exogenously added PGE_2 and the COX inhibitor indomethacin had no effect on wound repair. The levels of PGE_2 in cell culture supernatants at baseline and in response to wounding were lower than the concentration of PGE_2 required to significantly enhance 16HBE 14o$^-$ wound repair in Savla's model. Moreover, the addition of PGE_2 had no effect on the release of fibrinogen or FXIIIA at baseline or from wounded 16HBE 14o$^-$ cell monolayers. The differences in responses may be due to cell culture conditions. PGE_2 is a lipid-derived autacoid, therefore the lack of serum in the current model reduced the availability of phospholipid for phospholipase A2 activity, arachidonic acid production and subsequent PGE_2 synthesis.

Savla *et al.* reported that the EP-1 and EP-4 receptor subtypes were involved in the transduction of the stimulus from PGE_2 in 16HBE 14o$^-$ cells to promote wound repair. Interestingly, in the current study, mRNA expression of these receptors was not demonstrated in the same cell type. It is possible that the current culture conditions may have down-regulated expression of these receptors. COX-1 and COX-2 are similarly down-regulated in serum-free conditions. Conversely, 16HBE 14o$^-$ cells demonstrated mRNA expression of EP-2 and EP-3 receptors. There was no influence of time *post*-wounding on EP-2 mRNA expression in this cell type, whereas EP-3 was constitutively expressed and expression was increased in a time-dependent manner *post*-wounding, with levels of significance achieved at 10 hours. However, Savla *et al.* demonstrated that treatment of 16HBE 14o$^-$ cells with the EP-3 agonist enprostil resulted in neither stimulation nor inhibition of wound repair under the conditions used. Moreover, the same authors were unable to determine the importance of the EP-2 receptor subtype.

Thus, in order to determine whether EP-2 and EP-3 play a role in wound repair. the effects of the EP-2/EP-3 agonist misoprostol, or other available selective agonists could be investigated.

The tissue distribution and intracellular signalling pathways used by the subclasses of EP receptors are reported to differ substantially (Narumiya *et al.*. 1999). These differences may explain the wide array of effects that are induced by PGE_2 *in vivo*. For example, in cat trachea, PGE_2 acts as a dilator, but is a constrictor in guinea pig ileum (Gardiner, 1986). Similarly, PGE_2 may have dilator or constrictor effects on vascular smooth muscle (Walch *et al.*, 2001). The EP-2 receptor has been demonstrated to mediate the bronchodilatory effect of PGE_2 in mice (Fortner *et al.*, 2001; Hartney *et al.*, 2006; Sheller *et al.*, 2000). However, activation of EP-3 by PGE_2 was demonstrated to promote acute inflammation in a murine model (Goulet *et al.*, 2004). In terms of wound repair, there is no evidence to suggest that receptor subtypes EP-2 and EP-3 are involved. However, Savla *et al.* demonstrated that EP-4 mediated the effects of PGE_2 and stimulated wound repair following an increase in cAMP and EP-2 activation by PGE_2 is also associated with an increase in cAMP. Thus, there is a possibility that EP-2 may be involved in bronchial epithelial repair. The importance of EP-2 and EP-3 expression by the bronchial epithelium is yet to be established.

Chapter 7.

Preliminary Results Indicating Direction for Future Work

7. Preliminary results indicating direction for future work

7.1. Isolation of TF complex

7.1.1. Introduction

Initiation of the coagulation cascade requires the formation of a complex of TF with FVII. TF is the cellular receptor for zymogen FVII, facilitating its activation. FVII has minimal enzymatic activity in the unbound form and retains a zymogen-like conformation (Petrovan *et al.*, 2001; Zur *et al.*, 1982). Full catalytic activity of FVII is only acquired when present as part of a TF-FVII complex. Thus, the protease function of FVII *in vivo* is reported to be TF-dependent (Camerer *et al.*, 1996).

A second important feature of the initiation of coagulation is the interaction of the TF-FVII complex with FX. The Gla domain of FX provides critical phospholipid membrane binding and also interacts with an extended binding site in the C-terminal of TF and with the Gla domain of FVIIa (Ruf *et al.*, 1998). Together, the interaction of FX with TF-VIIa accounts for the binding energy that is required for the formation of the initial ternary TF-FVIIa-FXa complex (Shobe *et al.*, 1999). In this complex, the scissile bond of FX is presented to the active site of FVIIa and cleaved, generating product FXa. Thus, a ternary TF-FVIIa-FXa complex exists prior to the dissociation of product Xa (Kirchhofer *et al.*, 2001).

The formation of the TF-FVIIa-FXa in bronchial epithelial cells has not yet been investigated. In the current study, abundant expression of TF and FVII was demonstrated in 16HBE 14o$^-$ cells (*Chapter 6*) and FXa expression was demonstrated by use of FXa inhibitors (*Chapter 4*) and in preliminary experiments investigating gene repression (*Chapter 7*). Thus, it was of interest to investigate the formation of the ternary complex in response to mechanical wounding of bronchial epithelial cell monolayers.

7.1.2. Methods

7.1.2.1. Immunoprecipitation of TF complex

16HBE 14o$^-$ cells were cultured for 48 hours on 6-well cell culture plates until fully confluent and quiesced for 16 hours in 1 ml per well of serum-free MEM-ITS. Prior to experiment, MEM-ITS was refreshed and cell monolayers were maximally wounded, i.e. 4 horizontal scrapes and 4 vertical scrapes, as described in *section 2.2.5* and incubated at 37°C for 2, 5, 15, 30 and 60 minutes. Following incubation, supernatants were harvested and retained and cells were washed with 1X PBSc (PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Cells were incubated on ice for 30 minutes with PBSc containing 0.5 mM DTSSP (3,3′-Dithiobis[sulfosuccinimidylpropionate]), a water-soluble, thiol cleavable, homobifunctional and amine-reactive cross-linking agent. Cells were washed with 1 ml per well of TBS (50 mM NaCl, 50 mM Tris-HCl, pH 7.8) and 500 µl of lysis buffer (1X PBSc containing 1% Triton-x, 0.25% sodium deoxycholate, 0.5% SDS, 1X protease cocktail inhibitor (Calbiochem)) was added to each well. Once the cells had lifted, the cell lysates were extracted from the plate into 1.5 ml centrifuge tubes and centrifuged at 900 x *g* for 10 minutes at 4°C (ALC PM140R Centrifuge, Winchester Virginia, USA) to remove cell debris.

The Seize® X Protein A Immunoprecipitation kit (Pierce) was used to immunoprecipitate the TF.

7.1.2.2. Binding of antibody to immobilised protein A

To spin cup columns, 400 µl of the gel containing immobilised protein A was added and spin cup columns were placed inside 1.5 ml microcentrifuge tubes and centrifuged for one minute at 5000 x *g* (ALC PM140R Centrifuge, Winchester Virginia, USA). The flow-through was discarded and the spin cup columns returned to microcentrifuge tubes. The protein A gel was washed by adding 400 µl of binding/wash buffer (1X PBS containing: 0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M potassium phosphate and 0.01 M KCl, pH 7.4). The gel was resuspended by inversion and gentle shaking and

tubes were centrifuged for one minute at 5000 x *g* at room temperature and the flow-through was discarded. The wash step was repeated and spin cup columns were transferred into new microcentrifuge tubes. 40 µl of mouse anti-human TF. (1 mg/ml; American Diagnostica, Dundee, UK) was added to 400 µl binding/wash buffer in each spin cup column and tubes were incubated for 2 hours at room temperature with rotation. Tubes were centrifuged for one minute at 5000 x *g* at room temperature, spin cup columns were transferred into new microcentrifuge tubes and 500 µl binding/wash buffer was added to each tube. Tubes were inverted 10 times and centrifuged for one minute at 5000 x *g* at room temperature. The flow-through was discarded and the wash step was repeated twice more. Spin cup columns were transferred into new microcentrifuge tubes and 400 µl binding/wash buffer was added.

7.1.2.3. Cross-linking the bound antibody

To spin cup columns containing the bound antibody support, 25 µl of DSS (disuccinimidyl suberate; 25 mg/ml) was added and the tubes were mixed on a rotator for 60 minutes at room temperature. Following incubation, tubes were then centrifuged for one minute at 5000 x *g* and the flow-through was discarded. To spin cup columns, 500 µl of the ImmunoPure® IgG elution buffer pH 2.8 was added to remove IgG that was not covalently bound to protein A. The tubes were centrifuged for one minute at 5000 x *g* and the flow-through was discarded each time. This was repeated four additional times to quench the reaction and to remove excess DSS and uncoupled antibody. Spin cup columns were transferred into new microcentrifuge tubes and washed twice with binding/wash buffer, centrifuging for one minute at 5000 x *g* and discarding the flow through each time.

7.1.2.4. Antigen immunoprecipitation

To 400 µl of cell lysates (B/L, 2, 5, 15, 30 and 60 minutes *post*-wound) in 1.5 ml microcentrifuge tubes, 400 µl of binding/wash buffer and 125 µl of the cross-linked anti-TF protein beads was added and samples were rotated overnight at 4°C. 400 µl from each tube was transferred into new spin cup columns and centrifuged for one

minute at 5000 x *g*. The flow-through was discarded and 400 µl of anti-TF protein beads/lysate mixture was added to spin cup columns. Columns were centrifuged for one minute at 5000 x *g*, discarding the flow-through each time until all of the sample had been loaded into the spin cup columns. 500 µl of binding/wash buffer was added to the beads and the tubes were inverted 10 times then centrifuged for one minute at 5000 x *g* and the flow-through discarded. The wash step was repeated an additional two times and after the final wash, spin cup columns were placed into new microcentrifuge tubes and the wash step was repeated one additional time.

7.1.2.5. Elution of the immunoprecipitated antigen

The ImmunoPure® IgG elution buffer was neutralised by adding 10 µl of 1 M Tris pH 9.5 per 200 µl of elution buffer. To spin cup columns, 50 µl of the neutralised ImmunoPure® IgG elution buffer was added and mixed by inversion, then centrifuged for one minute at 5000 x *g*. This was repeated three additional times and all four fractions were retained for separate analysis.

7.1.2.6. SDS-PAGE

The resultant fractions from immunoprecipitation for TF were subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) in order to separate the proteins according to their size. For this purpose, a 10% resolving gel was used. Samples were run slowly through the stacking gel at 60 V, and then the voltage was increased to 90 V until the samples had reached the bottom of the gel. The separated proteins from the gel were transferred to 0.45 µm nitrocellulose membrane by semi-dry electrophoretic transfer (BioRad) at 250 mA for 60 minutes at room temperature. The nitrocellulose membranes were then blocked overnight in 1X PBS-2% ($^{v}/_{v}$) Tween-20 at 4°C to block non-specific binding sites.

7.1.2.7. Western blotting for TF

Membrane blots were washed three times for 10 minutes each in PBS-0.05% ($^{v}/_{v}$) Tween-20, at room temperature, with shaking. For the detection of TF, the primary antibody (mouse monoclonal anti-human TF, 1 mg/ml stock: American Diagnostica. supplied by Axis-Shield, Dundee, UK) was diluted 1 in 200 in 1X PBS-2% ($^{v}/_{v}$) Tween-20, 1% ($^{w}/_{v}$) BSA. To each membrane blot, 5 ml of the antibody solution was applied. Membrane blots were placed on parafilm in plastic trays and incubated with antibody for 1 hour at room temperature. Blots were washed three times for 10 minutes each in PBS-0.05% ($^{v}/_{v}$) Tween-20, at room temperature, with shaking. Secondary antibody (biotinylated rabbit anti-mouse $F(ab')_2$ fragment, Dako) was diluted 1 in 300 in 1X PBS-2% ($^{v}/_{v}$) Tween-20 1% ($^{w}/_{v}$) BSA and 5 ml of the antibody solution was applied to each membrane blot and incubated for 2 hours at room temperature. Blots were washed three times for 10 minutes each in PBS-0.05% ($^{v}/_{v}$) Tween-20 at room temperature, with shaking. Membranes were incubated in 40 ml SAB complex (StreptABComplex) for 45 minutes at room temperature. The SAB complex was prepared 90 minutes prior to use by adding 2 µl A (streptavidin) and 2 µl B (biotin-HRP) to 496 µl PBS and made up to 40 ml with PBS-2% ($^{v}/_{v}$) Tween-20 immediately before use. The blots were washed five times for 10 minutes each with PBS-0.05% ($^{v}/_{v}$) Tween-20. For chemiluminescent detection, Pierce West Pico SuperSignal substrate (Perbio, Northumberland, UK) was used. Activator and peroxidase solutions were added together in a 1:1 ratio and 5 ml was added to the membrane blot for 5 minutes at room temperature. Blots were transferred into plastic, zip-lock bags and excess substrate removed by blotting. Blots were placed in a cassette and exposed to X-ray films overnight and films were developed using the Kodak X-ray developer.

7.1.3. Preliminary results

7.1.3.1. Isolation of TF complex

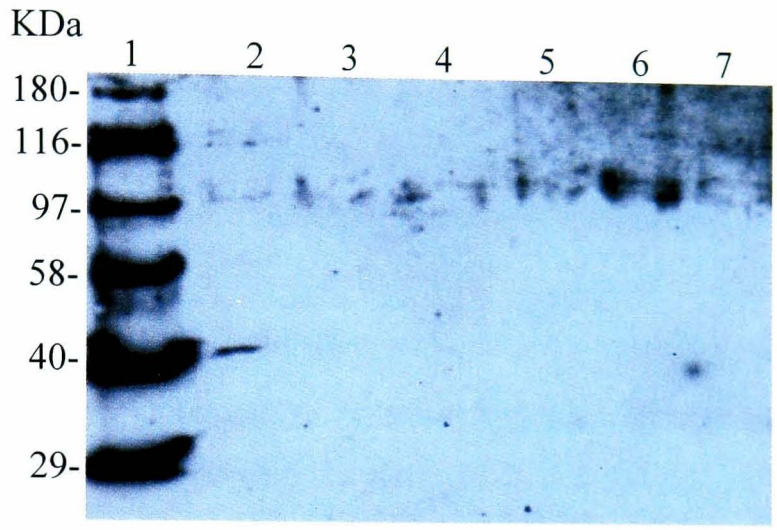

Figure 7.1. A representative image of the isolation of a TF complex.
Lane contents as follows: (1) Biotinylated Molecular Weight Marker (2) Unwounded cells (3) 2 minutes *post*-wound (4) 5 minutes *post*-wound (5) 15 minutes *post*-wound (6) 30 minutes *post*-wound (7) 60 minutes *post*-wound.

Preliminary data has demonstrated the presence of a TF complex that appears to increase with time following wounding. In the unwounded cells in *lane 2*, it is likely that the band at approximately 40 KDa is free TF (MW=47 KDa) and following wounding, this appears to form a high molecular weight complex around 97 KDa, whereby, its abundance is time-dependent following wounding and maximum abundance is achieved at 30 minutes *post*-wounding (*lane 6*).

7.1.4. Discussion

Preliminary data represents the novel observation of the formation of a TF complex in 16HBE 14o$^-$ cells in response to mechanical wounding. With respect to the molecular weight of the coagulation factors, it is likely that the bands at 97 KDa relate to the initial complex of TF-FVIIa (i.e. TF: 47 KDa and FVII: 50 KDa). It is possible that the high molecular weight band present in *lane 6* and 7 between 116-180 KDa may correlate to the ternary complex of TF-FVII-FXa (146 KDa), however, detection is negligible.

The presence of a band at approximately 40 KDa (*lane 2*), indicative of unbound TF may relate to full length TF protein that consists of a 219-amino acid extracellular region, a 23-residue transmembrane domain and a 21-residue intracellular region. Alternatively, it may represent alternatively spliced TF (asTF), a naturally occurring soluble TF isoform which was identified in HL-60 cells and is composed of 206 amino acids, containing most of the extracellular domain of full TF but lacking its membrane anchorage (Szotowski *et al.*, 2006). The fact that TF was demonstrated in unwounded cell monolayers supports the immunohistochemical (*section 3.4.1.1*) and RT-PCR (*section 6.4.1.3*) analysis that TF is constitutively expressed. It is evident that following mechanical wounding, there is rapid formation of a TF complex, which is required for initiation of coagulation. Conversely, TF mRNA is significantly increased over longer time frames of 10 hours, supporting the concept that following mechanical wounding, the initiation of coagulation is dependent on the release of preformed proteins as opposed to protein synthesis.

The increased formation of D-dimers in response to wounding (*section 3.4.2.3*) at 2 hours supports the notion that there is rapid formation of a TF complex prior to initiation of coagulation and that fibrin is rapidly generated and degraded *post*-wounding.

The initial aims of future work would be to improve the sensitivity of the method by optimising conditions such as to increase the loading volume and to use a lower percentage resolving gel (7.5%) to improve the resolution of high molecular weight proteins. With more time, it would have been useful to probe for FVII and FXa as well as TF. In order to confirm the presence of these coagulation factors in complex with TF, the same TF immunoprecipitates could be used, with subsequent immunostaining for both FVII and FXa. In addition to wounding, the effect of other relevant factors known to induce TF expression could be investigated at baseline and in wounded cell cultures. One such possibility could be endotoxin as this relates to excessive fibrin formation in acute lung injury (Schultz *et al.*, 2003) and ARDS (Idell, 2002). It would also be of interest to investigate whether or not endotoxin also enhances epithelial repair.

7.2. Histone deacetylation

7.2.1. Introduction

Regulation of gene expression is largely influenced by the architecture of DNA (de Ruijter *et al.*, 2003). In resting cells, DNA is tightly compacted to prevent accessibility of transcription factors and DNA forms a highly dynamic and organised complex with chromatin. Chromatin contains a nucleosome that is composed of an octamer of four core histones, i.e. an H3/H4 tetramer and two H2A/H2B dimers surrounded by 146 base pairs of DNA (Ito *et al.*, 2000; Strahl *et al.*, 2000). During activation of gene transcription, the compacted DNA is made available to DNA binding proteins *via* modification of the nucleosome (Ito *et al.*, 2000). The structure of chromatin is strongly influenced by post-translational modifications of the core histones. One such modification is histone acetylation.

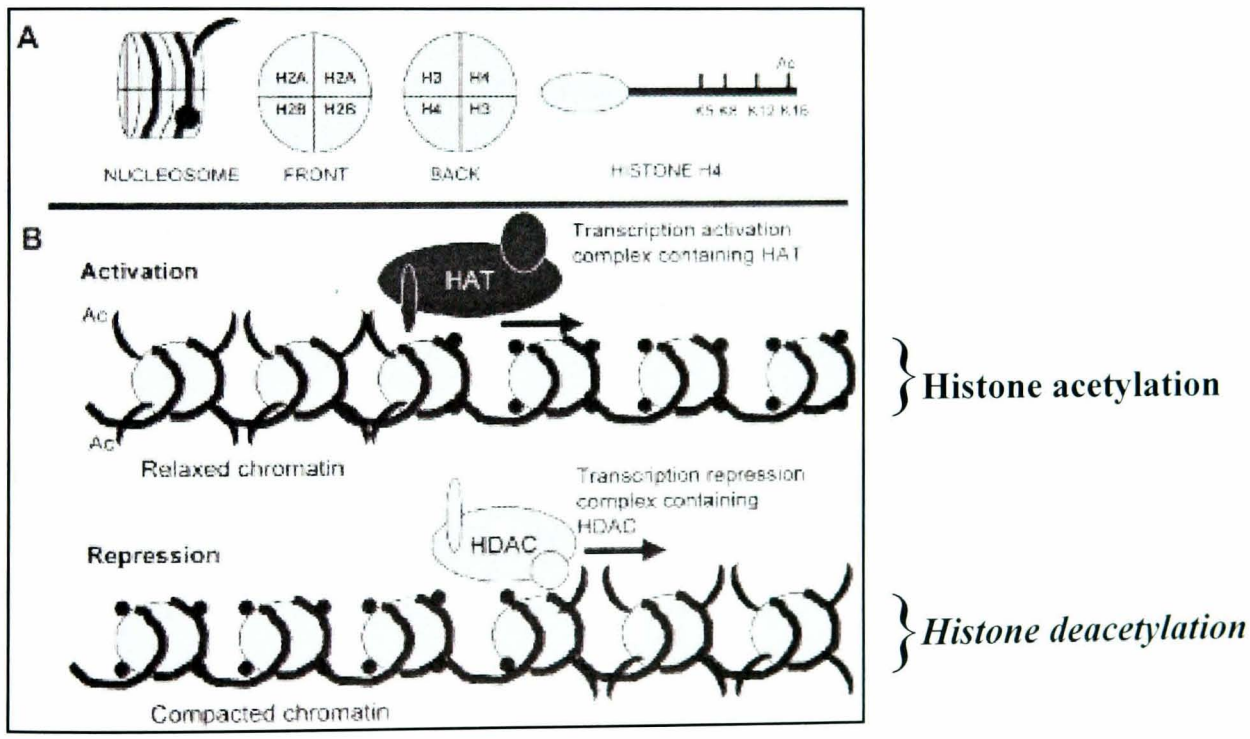

Figure 7.2. (A) Schematic representation of a nucleosome consisting of an H3/H4 tetramer and two H2A/H2B dimers. (B) Regulation of transcription by histone modification. The upper panel indicating histone acetylation depicts the addition of acetyl groups to core histones by HAT to activate gene transcription and the lower panel indicating histone deacetylation represents the removal of acetyl groups from core histone by HDAC resulting in repression of gene transcription.

Histone acetylation is a major modification that affects gene transcription and is controlled by histone acetyltransferases (HATs). Each core histone has a long terminal,

which is abundant in lysine residues that may be acetylated resulting in alterations of electrical charge of the core histones, leading to transformation of the chromatin structure from the 'resting' conformation that is closed to an activated open form (Roth *et al.*, 2001). This allows binding of TATA box-binding protein (TBP), TBP-associated factors and RNA polymerase II, which initiates gene transcription. In contrast, histone deacetylases (HDACs) remove the acetyl groups from hyperacetylated histones to counteract the effects of HATs and return histone to its basal state whereby gene expression is suppressed (Barnes *et al.*, 2005). Thus, acetylation of histones by HATs is associated with increased gene transcription, whereas hypoacetylation induced by HDACs termed 'histone deacetylation' is associated with suppression of gene expression.

HDACs play a major role in maintaining the balance between the acetylated and deacetylated states of chromatin. In humans, there are 18 potential deacetylase enzymes, HDAC1 to HDAC11 and silent information regulator (Sir)-like deacetylases, or sirtuins Sir1 to Sir7, which are responsible for the removal of acetyl groups and maintenance of the equilibrium of lysine acetylation in histones (Glozak *et al.*, 2005). HDACs are divided into two major classes: class I HDACs (HDACs 1, 2, 3, and 8) which are closely related to the yeast (*Saccharomyces cerevisiae*) transcriptional regulator protein RPD3 and localized to the nucleus; and class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) which are homologous to the yeast HAD-1 protein and are found in both the nucleus and cytoplasm (Barnes *et al.*, 2005). The sirtuins represent class III HDACs (Grozinger *et al.*, 2002). The sirtuin deacetylases contain a conserved 275 amino acid domain, which is unrelated to the other classes of HDACs and the sirtuin deacetylases function *via* a different mechanism that involves NAD as a substrate. Despite these structural and mechanistic differences, both the sirtuin deacetylases and HDACs are demonstrated to repress gene transcription at specific promoters or chromosomal domains by localised histone deacetylation (Grozinger *et al.*, 2002). HDAC11 is the sole member of class IV HDACs. It shares sequence similarity with the catalytic core regions of both class I and II enzymes but does not have strong enough identity to be placed in either class (Gao *et al.*, 2002).

Inflammatory lung diseases such as asthma are typically associated with increased expression of multiple inflammatory genes that are regulated by proinflammatory

transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1 (Barnes *et al.*, 1998). These inflammatory genes code for expression of cytokines, chemokines, enzymes that synthesise inflammatory mediators, mediator receptors and adhesion molecules (Barnes *et al.*, 2005). Many of these inflammatory genes are not expressed in normal cells under resting conditions, thus the increased expression of these proteins arises from enhanced gene transcription (Barnes *et al.*, 1998). Histone acetylation is an active process whereby minor changes in acetylases or deacetylases can markedly influence the overall HAT activity that is associated with inflammatory genes. Thus, hyperacetylation and increased gene expression may be a consequence of increased HAT activation or a reduction in HDAC activity or expression. Modifications in histone acetylation are targeted to regions of DNA associated with specific activator sites within the promoters of induced inflammatory genes (Urnov *et al.*, 2001). A study using bronchial biopsies from asthma patients has previously demonstrated a significant increase in HAT and a small reduction in HDAC activity compared with normal airways, thus favouring increased inflammatory gene expression (Ito *et al.*, 2002). A similar observation was demonstrated in alveolar macrophages obtained by BAL from asthmatic patients (Cosio *et al.*, 2004).

Corticosteroids are largely used in asthma therapy (Barnes, 1998) and are reported to suppress inflammation by switching off inflammatory genes (Barnes *et al.*, 2003). Alternatively, corticosteroids may suppress inflammation by increasing the synthesis of anti-inflammatory proteins such as annexin-1, secretory leukocyte peptidase inhibitor (SLPI), IL-10, IκB-α (inhibitor of NF-κB) and glucocorticoid-induced leucine zipper protein (inhibitor of both NF-κB and AP-1) (Ito *et al.*, 2000). Despite the fact that corticosteroids are highly effective in the control of asthma, the concern is that a proportion of patients are resistant to this therapy (Adcock *et al.*, 2003). Moreover, patients with chronic obstructive pulmonary disease (COPD) are largely unresponsive to corticosteroids (Barnes, 2000).

As well as histones, other transcription factors such as NF-κB are targets for acetylation and deacetylation, which thereby modulate their transcriptional activity (Barnes *et al.*, 2005). It was previously demonstrated in a study using the bronchial alveolar A549 cell line that activation of NF-κB induced by exposing the cell to inflammatory signals such

as IL-1β, TNF-α or endotoxin lead to acetylation of lysine residues on histone H4 which correlated to increased expression of the inflammatory mediator GM-CSF (Ito *et al.*, 2000). NF-κB is a major regulator of inflammatory genes, including cytokines, chemokines, inflammatory enzymes and adhesion molecules (Barnes *et al.*, 1997), and is therefore considered as a target for novel anti-inflammatory therapies (Karin *et al.*, 2004). A recent study demonstrated the effect of NF-κB inhibitors in the A549 cell line and in primary NHBE cells (Newton *et al.*, 2007). Stimulation of cells with IL-β1 and TNF-α induced NF-κB-dependent gene transcription, which was repressed by selective NF-κB inhibitors (IKK inhibitors). Inhibition of NF-κB-dependent gene transcription correlated with loss of expression of intercellular adhesion molecule (ICAM)-1, IL-6, IL-8, GM-CSF, regulated upon activation normal T cell expressed and secreted (RANTES), growth-related oncogene-α and MCP-1. In contrast, the corticosteroid dexamethasone was without effect on NF-κB-dependent gene transcription. Since IKK inhibitors had a powerful anti-inflammatory effect on the bronchial epithelium, it was reported that these inhibitors might be beneficial in situations of resistance to traditional corticosteroid therapy.

Inhibitors of HDAC activity (HDACIs) increase histone acetylation and thereby increase gene expression by preventing deacetylation (Moreira *et al.*, 2003). Trichostatin A (TSA) is a non-selective HDAC inhibitor which is known to alter gene expression by interfering with the removal of acetyl groups from histones and thereby altering the ability of DNA transcription factors to access the DNA molecules inside chromatin (Yoshida *et al.*, 2003). TSA has been demonstrated to overcome gene repression induced by HDACs by stimulating increased expression of inflammatory genes such as GM-CSF and IL-8 following exposure to inflammatory stimuli (Cosio *et al.*, 2004; Ito *et al.*, 2000; Tomita *et al.*, 2003). Moreover, the HDACIs TSA and sodium butyrate have both been demonstrated to stimulate the expression of t-PA in human umbilical vein endothelial cells (HUVECs) in a process that involved histone H4 acetylation (Arts *et al.*, 1995). The relevance of increased t-PA expression is that this mediator plays a key role in fibrinolysis by converting the zymogen plasminogen into the active enzyme plasmin, which mediates the breakdown of fibrin. The activity of t-PA is inhibited by PAI-1. The vascular endothelium plays an important role in regulating plasma t-PA activity by synthesising both t-PA and PAI-1 (Kooistra *et al.*,

1994). Mice that are deficient in t-PA have been demonstrated to have a reduced fibrinolytic capacity and thereby display extensive fibrin deposition (Carmeliet *et al.*, 1994). In this situation, HDACIs may be beneficial in restoring the balance between fibrinogenesis and fibrinolysis.

Gene repression may also occur *via* the less well documented process of DNA methylation. DNA methylation involves the addition of a methyl group to a section of DNA, which can lead to repression of gene transcription. It has recently been uncovered that there is tight epigenetic regulation involved in the expression of ADAM33 and expression of this gene has recently been demonstrated in the H292 bronchial epithelial cell line following demethylation of DNA at the ADAM33 promoter region known as the 'CpG island' (Holgate *et al.*, 2006). In bronchial epithelial cells, the CpG island of ADAM33 is hypermethylated, thus demethylation of DNA restores ADAM33 gene expression. ADAM33 has been identified as a susceptibility gene through genetic linkage analysis and association studies of families with asthma (Van Eerdewegh *et al.*, 2002) and until recently, expression of this gene was reported to be localised to smooth muscle, fibroblasts and myofibroblasts but not inflammatory cells, immune cells or the bronchial epithelium (Howard *et al.*, 2003; Van Eerdewegh *et al.*, 2002).

Since mRNA for fibrinogen and coagulation factors Xa and XIII was not detected in 16HBE 14o$^-$ cells (*Chapter 6*), it was of interest to investigate the possibility of gene repression using the HDACIs, TSA and sodium butyrate.

7.2.2. Methods

7.2.2.1. HDAC inhibition

16HBE 14o$^-$ cells were cultured for 48 hours on 6-well plates until fully confluent and quiesced for 16 hours in 1 ml per well of serum-free MEM-ITS. Prior to experiment, MEM-ITS was refreshed, 1 ml per well. One 6-well plate of cells was used for each condition. Final concentrations of 1 mM and 300 nM of the non-specific HDACIs sodium butyrate and TSA respectively, were added to wells and cells were incubated in the absence and presence of HDACIs for 48 and 72 hours. With the exception of the

unwounded cell controls, cells were wounded with maximum scrape damage (W8 = 4 horizontal wounds and 4 vertical wounds) and incubated for 12 hours at 37°C.

7.2.2.2. RNA Isolation

Following wounding and subsequent incubation for 12 hours at 37°C, the cell culture supernatants were removed and harvested and cells were washed with 500 µl per well of 1X PBS then trypsinised by incubating with 250 µl per well of 1X trypsin-EDTA for 5 minutes at 37°C. Once the cells had lifted, 250 µl per well of FBS was added to neutralise trypsin activity. The cell suspension from each well of the 6-well plate was combined and removed into 15 ml centrifuge tubes and centrifuged for 7 minutes at 670 x *g* (ALCPK120 Centrifuge, Winchester Virginia, USA) to create a cell pellet. RNA was then isolated from the cell pellets: See *section 6.3.1* for method.

7.2.2.3. One-Step RT-PCR for β-actin, FGC, FXa, and FXIII

See *section 6.3.9.1* for methods.

7.2.3. Preliminary results

7.2.3.1. mRNA expression of β-actin in the absence and presence of HDAC inhibitors

Expression of β-actin mRNA in the absence and presence of HDACIs in unwounded and wounded cell cultures by RT-PCR analysis was investigated.

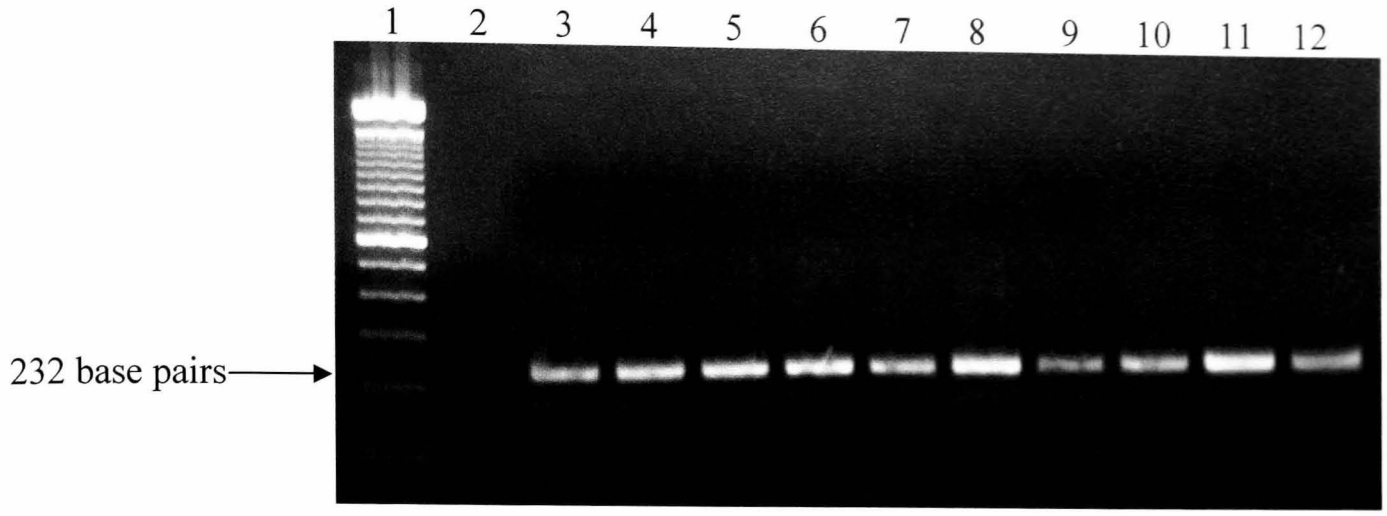

Figure 7.3. Representative gel demonstrating the effect of sodium butyrate and TSA on mRNA expression of β-actin in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (No RNA) (3) No drug; Baseline (unwounded cells): RNA extracted after 72 hours (4) No drug; Baseline (unwounded cells): RNA extracted after 48 hours (5) 1 mM sodium butyrate; Baseline (unwounded cells): RNA extracted after 72 hours (6) 300 nM TSA; Baseline (unwounded cells): RNA extracted after 72 hours (7) No drug; 12 hours *post*-wound: RNA extracted after 72 hours (8) No drug; 12 hours *post*-wound: RNA extracted after 48 hours (9) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 72 hours (10) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 48 hours (11) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 72 hours (12) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 48 hours.

RT-PCR amplification demonstrated that β-actin mRNA was expressed in 16HBE 14o$^-$ cells and was used as a suitable internal control for RT-PCR.

7.2.3.2. mRNA expression of FGC in the absence and presence of HDACIs

RT-PCR amplification in *section 6.4.3* indicated that fibrinogen mRNA was not detected in 16HBE 14o$^-$ cells. The fibrinogen primer pair was deemed valid since it was able to generate an abundant amplicon of 413 base pairs by RT-PCR, using RNA isolated from mouse liver. Furthermore, in the absence of RNA, no amplicon was generated by RT-PCR. However, evidence of fibrinogen protein expression in 16HBE 14o$^-$ cells was determined by immunoblot (*section 3.4.2.1*), therefore, gene repression was investigated by RT-PCR analysis of fibrinogen mRNA expression in the absence and presence of HDACIs.

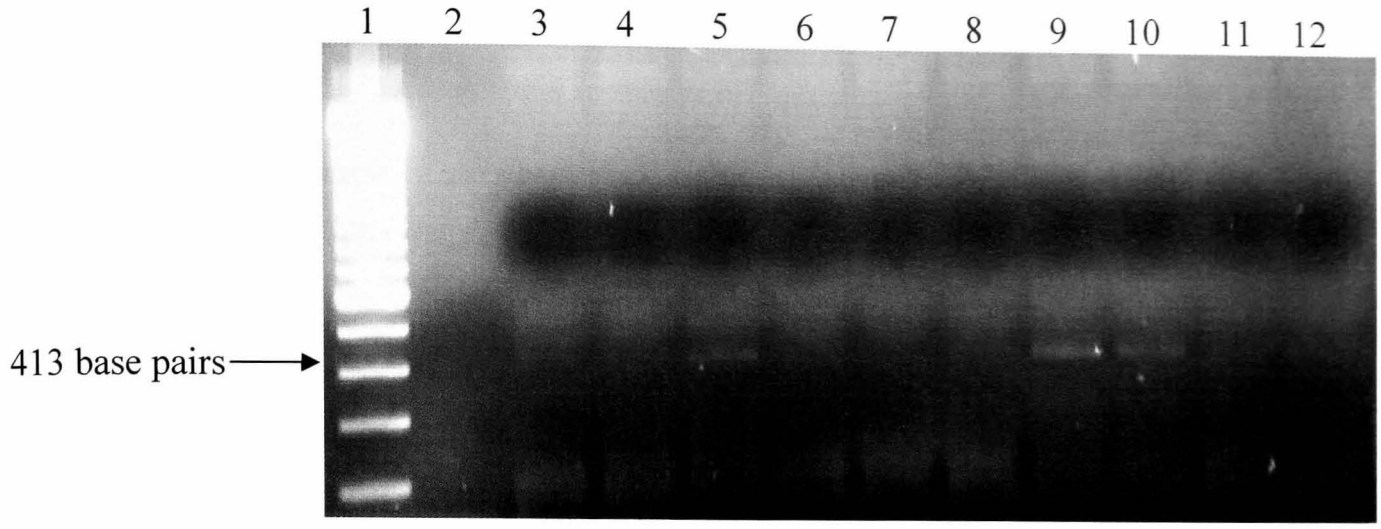

Figure 7.4. Representative gel demonstrating the effect of sodium butyrate and TSA on mRNA expression of FGC in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (No RNA) (3) No drug; Baseline (unwounded cells): RNA extracted after 72 hours (4) No drug; Baseline (unwounded cells): RNA extracted after 48 hours (5) 1 mM sodium butyrate; Baseline (unwounded cells): RNA extracted after 72 hours (6) 300 nM TSA; Baseline (unwounded cells): RNA extracted after 72 hours (7) No drug; 12 hours *post*-wound: RNA extracted after 72 hours (8) No drug; 12 hours *post*-wound: RNA extracted after 48 hours (9) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 72 hours (10) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 48 hours (11) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 72 hours (12) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 48 hours.

With reference to the unwounded cells in *figure 7.4*, RT-PCR analysis indicated that in the absence of HDACI, mRNA expression levels of FGC were low, resulting in extremely low abundance of FGC amplicons, regardless of time prior to RNA extraction. The addition of the HDACI, sodium butyrate had a positive effect on mRNA expression; this is evident in *lane 5,* whereby the abundance of the FGC amplicon is increased. The same pattern was apparent in the wounded cell monolayers; in the absence of drug, the low abundance of FGC amplicons depicted by *lanes 7 & 8* suggest that mRNA expression levels of FGC are very low, but the addition of sodium butyrate (*lanes 9 & 10*) appeared to enhance expression. This was particularly evident in cells that were incubated with sodium butyrate for 72 hours prior to extraction (*lane 9*). TSA however, had no effect on mRNA expression of FGC in the wounded or the unwounded cell monolayers.

7.2.3.3. mRNA expression of FXa in the absence and presence of HDACIs

RT-PCR amplification in *section 6.4.3* indicated that FXa mRNA was not detected in 16HBE 14o$^-$ cells. The FXa primer pair was deemed valid since it was able to generate an abundant amplicon of 430 base pairs by RT-PCR, using RNA isolated from mouse liver. Furthermore, in the absence of RNA, no amplicon was generated by RT-PCR. However, evidence that FXa is present in 16HBE 14o$^-$ cells was determined by inhibition of wound repair by two separate selective FXa inhibitors (*section 4.4.8.2*), therefore, gene repression was investigated by RT-PCR analysis of FXa mRNA expression in the absence and presence of HDACIs.

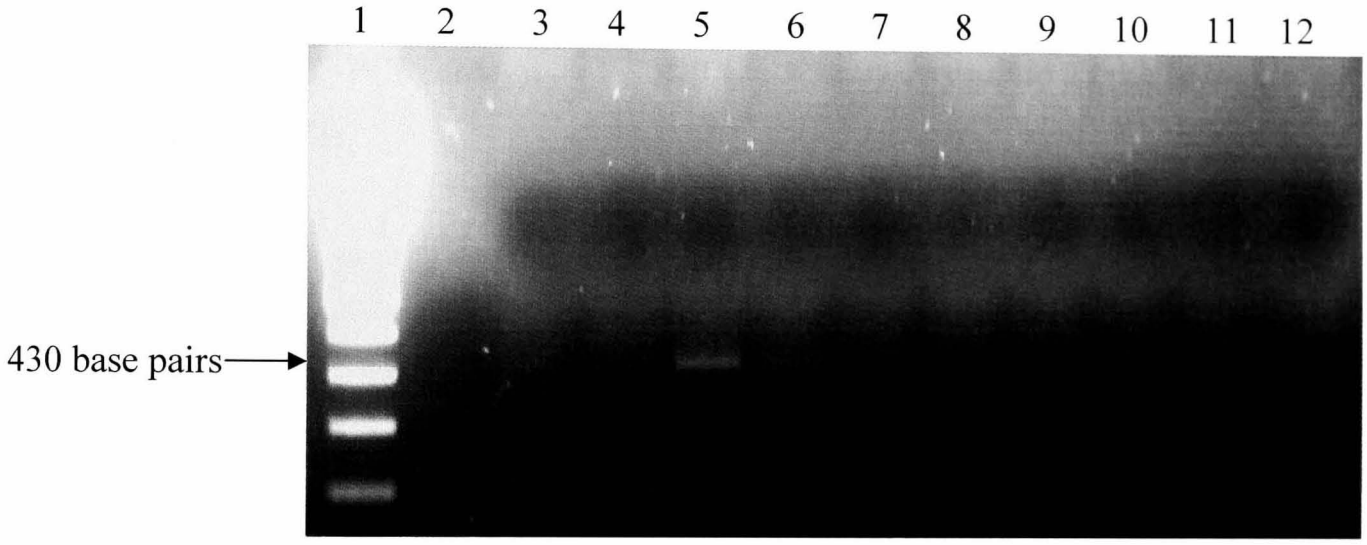

Figure 7.5. Representative gel demonstrating the effect of sodium butyrate and TSA on mRNA expression of FXa in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (No RNA) (3) No drug; Baseline (unwounded cells): RNA extracted after 72 hours (4) No drug; Baseline (unwounded cells): RNA extracted after 48 hours (5) 1 mM sodium butyrate; Baseline (unwounded cells): RNA extracted after 72 hours (6) 300 nM TSA; Baseline (unwounded cells): RNA extracted after 72 hours (7) No drug; 12 hours *post*-wound: RNA extracted after 72 hours (8) No drug; 12 hours *post*-wound: RNA extracted after 48 hours (9) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 72 hours (10) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 48 hours (11) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 72 hours (12) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 48 hours.

In the absence of HDACI, mRNA expression levels of FXa (demonstrated by *figure 7.5: lanes 3 & 4*) in the unwounded cells were almost undetectable. The addition of sodium butyrate enhanced expression. This was evident from the FXa amplicon present in *lane 5*, whereby the cells were incubated with the HDACI for 72 hours prior to RNA

extraction. However, this effect was not seen in the wounded cells and TSA had no effect on mRNA expression in the wounded or unwounded cell monolayers.

7.2.3.4. mRNA expression of FXIII in the absence and presence of HDACI

RT-PCR amplification in *section 6.4.4* indicated that FXIIIA mRNA was not detected in 16HBE 14o$^-$ cells. The FXIII primer pair was deemed valid since it was able to generate an abundant amplicon of 445 base pairs by RT-PCR, using RNA isolated from mouse liver (*figure 6.12*). Furthermore, in the absence of RNA, no amplicon was generated by RT-PCR. However, evidence of FXIIIA protein expression in 16HBE 14o$^-$ cells was determined by immunoblot (*section 3.4.2.2*) therefore, gene repression was investigated by RT-PCR analysis of FXIIIA mRNA expression in the absence and presence of HDACIs.

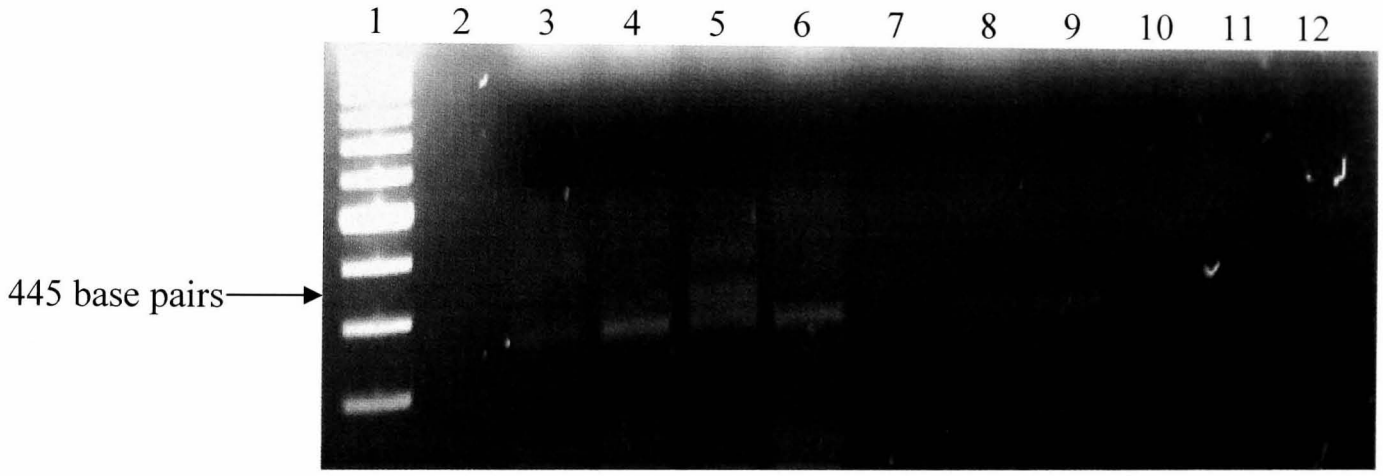

Figure 7.6. Representative gel demonstrating the effect of sodium butyrate and TSA on mRNA expression of FXIIIA in 16HBE 14o$^-$ cells.
Lane contents as follows: (1) 100 base pair ladder (2) Negative control (No RNA) (3) No drug; Baseline (unwounded cells): RNA extracted after 48 hours (4) No drug; Baseline (unwounded cells): RNA extracted after 72 hours (5) 1 mM sodium butyrate; Baseline (unwounded cells): RNA extracted after 72 hours (6) 300 nM TSA; Baseline (unwounded cells): RNA extracted after 72 hours (7) No drug; 12 hours *post*-wound: RNA extracted after 72 hours (8) No drug; 12 hours *post*-wound: RNA extracted after 48 hours (9) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 72 hours (10) 1 mM sodium butyrate; 12 hours *post*-wound: RNA extracted after 48 hours (11) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 72 hours (12) 300 nM TSA; 12 hours *post*-wound: RNA extracted after 48 hours.

RT-PCR amplification demonstrated that in the absence of HDACI, the level of mRNA expression of FXIIIA is low (as depicted by the FXIIIA amplicon of low abundance in *lane 4*), whereby RNA was extracted after 72 hours incubation.

With reference to the unwounded cells depicted by *lanes 3-6*; in the absence of HDACI at 48 and 72 hours (*lanes 3 and 4*), bands were present, but not in the position of 445 base pairs for full length FXIIIA. The presence of the HDAC inhibitor sodium butyrate resulted in 3 amplicons of different size, one of which was of the correct size of 445 base pairs and two others, which were around 400 and 500 base pairs. TSA did not appear to have any effect on the mRNA expression of FXIIIA compared to the no HDAC controls.

In the wounded cell cultures (*lanes 7-12*), it appeared that wounding decreased the level of FXIII mRNA expression (*lane 7*) compared to the unwounded cell cultures (*lane 4*) at 72 hours. In the absence of HDACIs, abundance of FXIII amplicons was low (*lanes 7 and 8*) but was slightly increased in the presence of the HDAC inhibitor sodium butyrate (*lane 9*). TSA did not appear to have any effect on the mRNA expression of FXIII compared to the no HDAC controls.

7.2.4. Summary of results

The HDACI sodium butyrate increased the mRNA expression of FGC in both the *un*wounded and wounded 16HBE 14o$^-$ cell monolayers. FXa mRNA expression was increased in the *un*wounded cells only in the presence of sodium butyrate. TSA had no effect on mRNA expression of neither FGC nor FXa mRNA in either the *un*wounded or wounded cell cultures under the conditions investigated. Finally, sodium butyrate generated multiple amplicons of FXIII of varying size in the *un*wounded 16HBE 14o$^-$ cells and TSA had no effect on mRNA expression of FXIII in either the *un*wounded or wounded cell cultures under the conditions investigated.

7.2.5. Discussion

Preliminary experiments investigating the effect of histone deacetylase inhibitors on fibrinogen, FXa and FXIII expression suggest that these genes are repressed in 16HBE $14o^-$ cells, as mRNA expression was increased in the presence of the HDACI sodium butyrate. In all cases, amplicons were most abundant when RNA was extracted 72 hours after incubation with sodium butyrate suggesting that HDAC inhibition was *time*-dependent.

In the absence of sodium butyrate, unwounded 16HBE $14o^-$ cell layers were demonstrated to express FXIII mRNA after 72 hours of culture, however its abundance was low. Previous experiments demonstrated that FXIII mRNA transcripts were not detected in 16HBE $14o^-$ cells (*section 6.4.4*). However, for the purpose of that experiment, the RNA was extracted immediately, with no prior incubation period. This suggests that the levels of mRNA expression of FXIII may be dependent upon duration of culture, prior to RNA extraction.

Following incubation of confluent monolayers of 16HBE $14o^-$ cells with sodium butyrate, three FXIII amplicons of approximately 500 base pairs, 445 base pairs (correct size) and 400 base pairs were obtained (*figure 7.6*). Since these amplicons were generated using a single primer pair, it is possible that three alternatively spliced variants of FXIII mRNA are encoded by a single gene. Another possibility is that genomic DNA is susceptible to environmental changes, giving rise to large missense mutations. In a previous study, molecular analysis of the FXIIIA gene in two unrelated FXIII-deficient individuals revealed three splice site mutations; a g-->a at the exon 6 acceptor splice site, a g-->a at the exon 7 donor splice site and a coding sequence T-->G at the exon 8 donor splice site (Anwar *et al.*, 1998). FXIIIA mRNA expression in these patients was also investigated and it was demonstrated that each mutation gave rise to multiple transcripts which varied in their relative abundance (Anwar *et al.*, 1998). It is possible that alternatively spliced variants of FXIII may relate to its various functional roles, including fibrin cross-linkage, wound repair and cross-linkage to fibronectin to anchor the fibrin clot to the site of injury. Another role performed by FXIII is to prevent spontaneous fibrinolysis by incorporation of α1-anti-plasmin into the fibrin clot. In

order to definitively identify the sequence of the three amplicons produced by RT-PCR as seen in *lane 5* of the gel shown in *figure 7.6*, it would be necessary to perform RT-PCR reactions containing (1) forward primer only (2) reverse primer only and (3) both primers, in order to ascertain that the three amplicons present are not a false positive result, and are the result of amplification of three unique mRNAs transcribed from one section of genomic DNA. mRNA purification and subsequent sequence analysis would elicit the identity of the three aforementioned amplicons.

The HDACI sodium butyrate exerts a very broad range of effects on many biological pathways *via* its inhibitory action on HDACs. Sodium butyrate has anti-inflammatory properties and is known to exert immune suppression in part by suppressing NF-κB activity (Joseph *et al.*, 2004; Segain *et al.*, 2000). Several investigators of histone acetylation recognised that to increase histone acetylation, either the activity of HAT must be increased, or conversely, the activity of HDAC be inhibited. The latter was found to be the mode of action of sodium butyrate (Boffa *et al.*, 1978; Candido *et al.*, 1978; Sealy *et al.*, 1978; Vidali *et al.*, 1978). Sodium butyrate inhibits most HDACs with the exception of class III HDACs representing the sirtuin (Sir) family, which includes yeast (Sir2) and class II HDAC6 and -10, however, the binding site by which sodium butyrate inhibits HDAC activity has not yet been established (Davie, 2003; Li *et al.*, 2006). During inhibition of HDAC activity, HAT activity continues, which results in histone hyperacetylation. Histones, however, are not the only substrates of these enzymes. High mobility group (HMG) chromosomal proteins are acetylated. HMG proteins are common to all eukaryotes and bind DNA in a non-sequence-specific manner to promote chromatin function and gene regulation. They interact directly with nucleosomes and are reported to be modulators of chromatin structure. HMG-1(Y) has a role in the transcription of many genes involved at different steps in the metastatic cascade and has been linked with cancer in human and animal models (Evans *et al.*, 2004). However, HMGs are also important in activating a number of regulators of gene expression, including p53, a transcription factor that regulates cell cycle and hence functions as a tumour suppressor (Sterner *et al.*, 1979). Studies reveal that among the fatty acid HDACIs, sodium butyrate is the most effective in inhibiting HDAC activity and repressing the expression of specific genes (Davie, 2003).

Under the conditions investigated, TSA had no effect on the expression of fibrinogen, FXa or FXIII. TSA is structurally unrelated to sodium butyrate, however it has previously been shown to be a potent and specific inhibitor of histone deacetylase at lower concentrations than sodium butyrate (Arts *et al.*, 1995; Yoshida *et al.*, 1990). In a study involving stimulation of t-PA gene expression by TSA in HUVECS, 1 μM TSA was determined to be the optimal concentration required for maximal induction of t-PA expression. These preliminary experiments only included one concentration of 300 nm TSA, therefore it would be necessary to investigate a concentration range of TSA in order to optimise conditions. However, following a previous study using a human lymphoid cell line, it was reported that only 2% of genes were regulated by TSA. Similarly, a study involving the effect of TSA in gastric cancer cells demonstrated that of the 2400 genes, only 5% were regulated (Glaser *et al.*, 2003). Moreover, TSA as well as other HDACIs are reported to have the potential to both up-regulate and down-regulate gene expression (Glaser *et al.*, 2003; Wang *et al.*, 2007).

Both sodium butyrate and TSA are described as non-selective inhibitors of HDACs (Barnes *et al.*, 2005; Davie, 2003; Yoshida *et al.*, 2003). Moreover, there is evidence that different HDACs target different patterns of acetylation and regulate different genes (Peterson, 2002). Thus, for future experiments, it would be of interest to investigate exactly which HDACs are involved in repression of coagulation factor expression. This would be achieved by RT-PCR analysis of coagulation factor mRNA expression in the absence and presence of highly specific HDACIs.

Although sodium butyrate enhanced the level of mRNA expression of fibrinogen, FXa and FXIII, the abundance of amplicons was low. Previous studies have indicated that the expression of HDACs 1-3 is affected by cell density (Dangond *et al.*, 2001; Gray *et al.*, 1998). It was demonstrated in the hepatocellular carcinoma (Hep 3B) and human prostate adeno-carcinoma (PC3) cell lines that the level of mRNA expression was decreased at high cell densities and in the case of HDAC3, mRNA expression levels were almost undetectable. It was observed that as cells were actively growing, treatment with sodium butyrate upregulated expression of HDACs 1-3. However, the response to the HDACI was reduced at higher cell densities particularly in the case of HDAC2 (Dangond *et al.*, 1998; Dangond *et al.*, 2001). In the current study, HDACIs were added

to *confluent* cell cultures prior to incubation, therefore it is possible that the response was reduced due to high cell density. For future experiments it would be of interest to add the HDACIs in a time-course during cell growth. Equally, it would be interesting to investigate the mRNA expression of HDACs at different cell densities of 16HBE 14o$^-$ cells.

Experiments involving neutralising antibodies have demonstrated that fibrinogen and FXIII are *essential* to wound repair. Since these genes are repressed it would be beneficial to target the specific HDACs in order to activate gene transcription and increase their expression. First it would be necessary to identify which HDACs are involved. This could be achieved by HDAC-specific chromatin immunoprecipitation (ChIP) analysis of HDACs bound to the promoter region of the coagulation factors, then to develop specific HDACIs to target them. ChIP analysis has recently been used to demonstrate the down-regulation of TF expression by HDAC3 (Wang *et al.*, 2007). Local production of coagulation factors to support bronchial epithelial repair may be important in asthma when there is reduced or no plasma exudation (refer to *Chapter 8* for more detail). However, it is becoming increasingly evident that the inflammation in asthma is largely driven by the increased expression of multiple inflammatory genes *via* activation of proinflammatory transcription factors, such as NF-κB and AP-1, resulting in acetylation of core histones and thus increased expression of inflammatory mediators. Thus, the challenge would be to target HDACs that would increase expression of coagulation factors without switching on expression of inflammatory mediators.

HDACs and HATs are known to bind to repressor and activator proteins, which are implicated in tumorigenesis and are therefore considered to play a central role in a wide variety of human cancers (Dangond *et al.*, 2001). Recent studies have revealed that HDACs are structurally and functionally diverse, therefore, it is recognised that inhibitors that are specific to individual enzymes must be developed as more promising agents for cancer therapy. Novel TSA/TPX hybrids have been synthesised, which will serve as a basis for developing enzyme-specific HDAC inhibitors (Yoshida *et al.*, 2003). Thus, the goal of developing specific HDAC inhibitors for coagulation factors is not unobtainable.

Chapter 8.

General Discussion

8. General discussion

Animal models have previously indicated that normal bronchial epithelial repair is rapid and supported by the formation of a provisional fibrin matrix that is exclusively plasma-derived. However, this study has demonstrated for the first time that bronchial epithelial cells in culture are a source of coagulation factors and are able to initiate coagulation in the *absence* of plasma. Two lines of evidence point to the capacity of these cells to generate a cross-linked fibrin matrix in the absence of plasma. Namely, the detection of fibrinogen and FXIIIA in cell culture supernatants *post*-wounding and the formation of D-dimer fragments indicative of sequential fibrinogenesis and fibrinolysis. Furthermore, blocking antibody experiments indicate that locally produced coagulation cascade proteins are *essential* for normal epithelial repair and the formation of insoluble cross-linked fibrin matrices is independent of plasma proteins providing evidence that repair of the wounded bronchial epithelium is *autonomous*.

It is evident that initiation of coagulation is rapid in the current model since a significant release of D-dimers was evident at 20 minutes. However, RT-PCR analysis in *Chapter 6* demonstrated a *time*-dependent increase in TF mRNA that was only significant at 10 hours *post*-wounding. Thus, with respect to the time frame of mRNA expression of TF, it would appear that gene transcription is not responsible for the rapid induction of TF-initiated coagulation in response to wounding. The likely concept is that TF is upregulated by a process of de-encryption. TF encryption describes the suppression of TF procoagulant activity on the cell surface. TF may become decrypted in response to stimuli such as freezing and thawing of cells and in the presence of proteases (Bach *et al.*, 1990; Le *et al.*, 1992), which may explain why TF is constitutively active in the current model. However, the upregulation of TF at the wound edge is likely to be the cause of further decryption of TF in response to cell damage. In the plasma membrane of quiescent cells, phosphatidylserine, a major phospholipid, is localised on the inner leaflet of the bilayer (Zwaal *et al.*, 1997); and an increase in cytosolic calcium of the same magnitude that decrypts TF procoagulant activity also disrupts phosphatidylserine asymmetry. The relevance of phosphatidylserine availability is that this phospholipid accelerates coagulation reactions on membrane surfaces (Hathcock *et al.*, 2005; Kunzelmann-Marche *et al.*, 2000). Interestingly, immunohistochemical staining in

Chapter 3 demonstrated the expression of TF localised in vesicles within cells at the wound edge. Thus, in response to wounding, it is likely that these vesicles are translocated to the plasma membrane to increase the phospholipid availability for TF procoagulant activity and to initiate coagulation. This evidence supports the concept that TF in part is pre-formed and stored in vesicles and may therefore be readily available to support repair when cells are injured.

Initiation of coagulation and PAR activation are believed to be inseparably linked (Chambers *et al.*, 2002). The coagulation factor proteases FVIIa, FXa and thrombin are the most thoroughly established activators of PARs. Coagulation factors most efficiently activate PARs when they are localised at the cell surface. For example, FVII and FXa effectively activate PARs when they are bound to the transmembrane TF receptor to form the transient ternary TF:FVIIa:FXa complex (Bunnett, 2006). In response to extravascular injury, active TF may initiate the coagulation cascade or alternatively trigger cell signalling *via* PAR activation. It is now considered that TF:FVII-mediated coagulation and cell signalling involve distinct cellular pools of TF and that signalling TF refers to the encrypted form and de-encryption of TF is necessary to promote coagulation (Ahamed et al., 2006).

Data generated from immunoblots in *Chapter 5* demonstrated that activation of PAR-1 and PAR-2 by synthetic peptide agonists lead to an increase in the release of fibrinogen and FXIIIA from 16HBE 14o$^-$ cells. In response to wounding alone, the release of fibrinogen and FXIIIA was also demonstrated. Thus, in the absence of exogenous PAR agonists, it is possible that following wounding of 16HBE 14o$^-$ cells, the release of fibrinogen and FXIIIA may have been a result of coagulation, or alternatively due to activation of both PAR-1 and PAR-2 by proteases of the coagulation cascade. However, when anti-TF was employed to block initiation of the coagulation cascade and wound repair (*Chapter 4*), there was an accumulation of fibrinogen in cell culture supernatants which signified that fibrinogen was released from 16HBE 14o$^-$ cells and NHBE cells but not consumed in the wound repair process. In this case, activation of PARs by coagulation cascade proteases was ruled out, implying that other endogenous proteases were responsible for the observed responses. It was suggested that MMP-1 and human airway trypsin (HAT) released from wounded epithelial cells may have been likely

candidates for the activation of PAR-1 and PAR-2 respectively, as proposed in the scheme represented in *figure 8.1*.

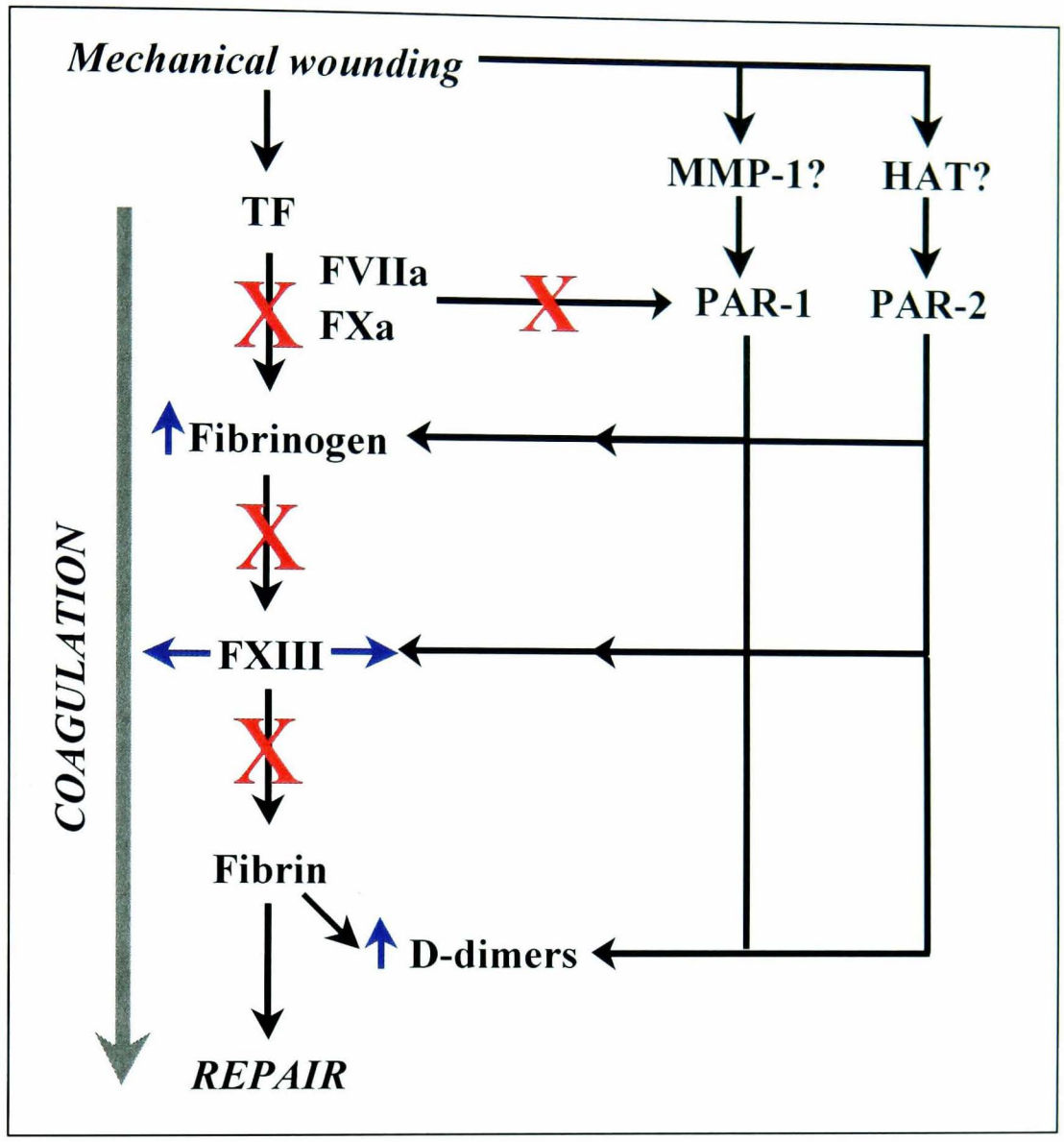

Figure 8.1. A schematic representation of the proposed mechanism of fibrinogen accumulation following blockade of the coagulation cascade. Mechanical wounding of 16HBE 14o$^-$ cells leads to the release of coagulation factors as a result of either initiation of coagulation or PAR activation. Supernatants demonstrated an accumulation of fibrinogen despite blockade of the coagulation cascade using a neutralising TF antibody. This may due to the release of MMP-1 and HAT (human airway trypsin) from wounded 16HBE 14o$^-$ cells and activation of PAR-1 and PAR-2 respectively.

Due to the highly simplified nature of the current model, the associated limitations are recognised. The epithelial cell monolayer displayed by the 16HBE 14o$^-$ cell line remains undifferentiated, unlike the bronchial epithelium *in vivo*. With more time, it would have been beneficial to develop a model of NHBE cells cultured on transwells at the air liquid interface in order to achieve a fully differentiated epithelium. Once established, the same experiments, including immunoblot analysis of coagulation factors released into supernatants and wound repair assays could have been employed.

However, both the serum-free nature of the current culture conditions and the lack of other contributory influences such as the underlying mesenchyme, growth factors and sensory neuropeptides have uncovered a previously unrecognised role bronchial epithelial cells in the local production of coagulation factors that have the capacity to generate a provisional fibrin matrix to support epithelial cell migration and are functionally important in normal wound repair.

Microvascular hyperpermeability and oedema formation are common features of inflammation. In patients with allergic asthma, inhalation of allergen produces both immediate and late-phase inflammatory responses, including the increased expression of various pro-inflammatory chemokines, cytokines, growth factors, lipid mediators, adhesion molecules and receptors for the same inflammatory mediators, during which plasma is exuded from the airway microcirculation (Greiff *et al.*, 2003; Liu *et al.*, 1991; Svensson *et al.*, 1995). Until this study, it had been assumed that coagulation factors were solely derived from plasma exudation in the inflamed airways, however, the local release of coagulation factors from bronchial epithelial cells in the absence of plasma, as demonstrated in the current study, may be important in the repair of the normal healthy bronchial epithelium as well as in asthmatic patients undergoing treatment, when plasma exudation may not follow epithelial injury.

Treatment of asthma is divided into two categories, one for attenuating the chronic inflammation using corticosteroids and the other for the relief of symptoms of airflow obstruction using bronchodilators such as β-adrenoceptors, theophylline, antileukotrienes and anticholinergic agents (Bardin *et al.*, 1992). In patients with asthma, inhaled corticosteroids have been shown to suppress the increased microvascular permeability and plasma exudation into the airway lumen as determined by measurements of concentrations of high molecular weight proteins such as α_2-macroglobulin in sputum and BAL fluid (Kanazawa *et al.*, 2002; Schoonbrood *et al.*, 1995). In addition, Schoonbrood *et al.* demonstrated that inhaled corticosteroid therapy inhibited plasma exudation without affecting local protein synthesis. Moreover, a study of the effect of prednisone in patients with severe persistent asthma indicated inhibition of plasma exudation with no significant change in levels of fibrinogen in the airways,

suggesting the likelihood of a local cellular source of fibrinogen (Pizzichini *et al.*, 1999).

Although, anti-asthma therapies are effective in suppressing inflammation and reducing oedema formation by inhibiting plasma exudation, they do not prevent the occurrence of epithelial injury. However, the effect of corticosteroids on bronchial epithelial repair is controversial since there is a report that demonstrates that these agents enhanced the integrity of the bronchial epithelium in an *in vitro* study using bronchial biopsy specimens (Vignola *et al.*, 2001) and a conflicting report which demonstrates that corticosteroids decreased the rate of cell migration following mechanical injury in a bronchial epithelial cell line (Dorscheid *et al.*, 2006). The bronchial epithelium is in contact with, and continually damaged by infectious agents that are frequently inhaled from the environment. Thus, in the absence of plasma, the local release of coagulation factors to promote bronchial epithelial repair may be functionally important.

In animal airways, steroid treatment is reported to induce indirect anti-vascular permeability effects *via* inhibition of inflammation (Persson *et al.*, 1998). The same research group demonstrated that other anti-asthma drugs including β-agonists, chromones and theophyllines reduce plasma exudation in inflammatory challenge-induced guinea pigs (Erjefalt *et al.*, 1991; Erjefalt *et al.*, 1991; Persson *et al.*, 1986). In support of this, a study demonstrated that the selective β_2-adrenergic receptor agonist formoterol, which is known to have an anti-oedema effect, reduced plasma exudation by inhibiting endothelial gap formation in a rat model (Baluk *et al.*, 1994). Similarly, treatment of rats with dexamethasone significantly reduced ovalbumin-induced plasma exudation (Damazo *et al.*, 2001).

TF-bearing cells are believed to be involved in the initiation of fibrin clot formation, while the expansion of the fibrin clot is dependent on the propagation of coagulation to bulk plasma (Ovanesov *et al.*, 2005). Thus, the release of coagulation factors from bronchial epithelial cells and subsequent formation of fibrin to support local bronchial epithelial repair is beneficial, provided that the fibrin clot is degraded after repair is complete. Efficient fibrin turnover is evident in the current study by the presence of D-dimers *post*-wounding. However, under pathological conditions, the high level of TF

expression by activated bronchial epithelial cells may be detrimental and propagation of coagulation into exudated plasma could ultimately lead to excessive fibrin deposition and thrombus generation, as demonstrated in conditions such as ALI (Idell, 2003). ARDS, a form of ALI (Idell, 2002), and *status asthmaticus* (Wagers *et al.*, 2004). Therefore, these conditions represent a target for *anticoagulant* therapy.

Anticoagulant therapy has already proven to be successful in the treatment of patients with sepsis and there is growing literature that suggests the possibility for use of selective anticoagulants for lung protection in conditions such as ALI. ALI arises from an imbalance in the lung characterised by enhanced coagulation and reduced fibrinolysis. However, it is recognised that the use of anticoagulant therapy to restore the abnormalities in coagulation and fibrinolysis may have a detrimental effect on pulmonary host defence since activation of coagulation not only leads to fibrin formation but is also associated with the modulation of inflammatory activation to produce protective anti-inflammatory cytokines (Schultz *et al.*, 2003). Thus, the benefit of anticoagulant therapy for ALI remains to be established.

Anticoagulant therapy with pentasaccharides, low molecular weight heparins (LMWHs), and unfractionated heparin (UFH) has been used for more than six decades. These compounds are indirect inhibitors of FXa, requiring the presence of antithrombin for an antithrombin effect. For many years, several direct FXa inhibitors have been in preclinical development but have not yet reached the stage of advanced clinical trials (Schulman, 2003). The two selective FXa inhibitors that were employed in the current study may be of potential use as an anticoagulant therapy in *status asthmaticus* for example, as they would overcome the problem of excessive fibrin formation in addition to enhancing bronchial epithelial repair.

In support of the concept that coagulation and PAR activation are inseparably linked (Chambers *et al.*, 2002), activation of PAR-1 and PAR-2 by synthetic peptide agonists was demonstrated to enhance fibrin formation and bronchial epithelial repair in the current study. There is accumulating evidence to suggest that proteases and their receptors play a fundamental role in inflammation. During inflammation and injury, there is upregulation and generation of many proteases that can activate PARs and the receptors themselves are often over-expressed in inflamed tissues. Administration of

selective PAR agonists and proteases can induce signs of inflammation as demonstrated by models of PAR-deficient mice, which have implicated PARs in the development of asthma.

In view of the emerging role of proteases and PARs in inflammation, there is considerable interest in the use of antagonists and agonists of these receptors for the treatment of a variety of diseases, including asthma, however, the lack of antagonists has impeded investigations of the role of PARs in experimental models of human disease. Tissue fibrosis is predominantly mediated *via* PAR-1 (Chambers *et al.*, 2002; Sokolova *et al.*, 2007). Since PAR-2 is the only subtype that is reported to be cytoprotective in the airway and, in this study was shown to enhance bronchial epithelial repair, it may beneficial in the future to develop an aerosolised specific PAR-2 agonist to promote repair of the damaged epithelium in asthmatics.

Although PAR-2 receptors have been implicated in inflammatory responses in animal models *in vivo* (Sokolova *et al.*, 2007), PAR-2 agonists did not induce an overt inflammatory response in mice (Henry, 2006). The response to inhaled PAR-2 agonists will depend on the level of expression of PAR-2 receptors in the disease setting and subsequent activation of cell signalling pathways leading to the generation of PGE_2, which is responsible for many of the beneficial effects of PAR-2 activation. The prostanoid receptors EP-1 and EP-4 are associated with bronchial epithelial repair (Savla *et al.*, 2001), while the EP-2 and EP-3 receptors may be effective in producing bronchodilation and opposing allergen-driven inflammation. Thus, the increase in EP-3 receptor expression observed *post*-wounding in 16HBE $14o^-$ cell layers supports the notion that PAR-2 agonists may promote anti-inflammatory effects as well as bronchial epithelial repair.

Activation of the PAR-2-PGE_2-EP receptor axis in animal models of airway disease and in isolated human cells and tissues is demonstrated to produce a series of bronchoprotective effects including bronchorelaxation and anti-inflammatory effects (Henry, 2006). However, it is presently unclear whether these beneficial effects will be translated through to the treatment of human lung diseases. Moreover, it is unclear as to which component(s) of the PAR-2-PGE_2-EP receptor axis should be the pharmacological target. Given that the majority of the bronchoprotective effects are

mediated *via* PGE_2, it has been suggested that a better pharmacological approach may be to administer inhaled PGE_2 rather than a PAR-2 peptide agonist. However, the disadvantage of this approach is that activation of irritant receptors within the upper airways and the induction of reflex bronchoconstriction and cough may occur as a consequence (Tilley *et al.*, 2003). The current concern with the PAR-2 peptide agonists used in the majority of studies is that they have relatively low potency (micromolar range) and they are susceptible to degradation by aminopeptidases, which limits their usefulness in whole animal studies. However, a study has demonstrated that substitution of the N-terminal serine with a furoyl group in PAR-2 peptide agonists dramatically enhanced the agonistic activity and decreased degradation by aminopeptidase leading to the development of a more potent PAR-2 peptide (Kawabata *et al.*, 2004).

Effective treatments for asthma are available, however, morbidity and mortality rates are not improving. Many attempts have been made to improve existing therapies rather than to develop new ones. For example, long acting inhaled β_2-agonists were developed after oral β_2-agonists and were considered to be more effective and inhaled glucocorticoids were developed as they have fewer side effects than the oral formulation. Current asthma therapy is delivered predominantly by inhalation.

Decade	Drugs currently available	Drugs in development	Therapeutics that have failed to date
1930s	Theophylline		Neuropeptide antagonists
1950s	Anti-cholinergics		PAF antagonists
1960s	Short-acting B_2 agonists Inhaled and oral corticosteroids	Anti-TNF	Thromboxane inhibitors
1970s	Cytotoxics and immunosuppressants*		Bradykinin antagonists
1980s	Long-acting B_2 agonists	Type IV PDE inhibitors†	Anti-IL-5 mAb hr IL-12
1990s	Leukotriene receptor antagonists		hr IFN-γ
2000s	Anti-human IgE mAb		hr IL-10 Soluble IL-4 receptor IL-4 double mutein Allergen-specific IL VLA-4 antagonists P-selectin mAb Antihistamines Mast cell stabilisers

Table 8.1. A summary of the drugs that are currently available for asthma, therapies that have failed and drugs that are currently in development. *Rarely used and efficacy uncertain; †mostly targeted for COPD; PDE: phosphodiesterase; hr: human recombinant; mAb: monoclonal antibody; VLA: very late antigen.
Adapted from the review by Holgate *et al.* (2007).

For patients with mild-moderate asthma, regular inhalation of a low dose of inhaled corticosteroid is recommended; and for those with severe asthma that is not controlled on a low dose of inhaled corticosteroid alone, regular treatment with a long acting inhaled β_2-agonist or leukotriene receptor antagonist is prescribed in addition (Holgate *et al.*, 2003). Cortisone was first demonstrated to cause dramatic improvements in patients with rheumatoid arthritis in 1949; and since then, corticosteroids became established as the most potent anti-inflammatory agents in the pharmacotherapy of chronic inflammatory diseases such as asthma (Horvath *et al.*, 2006). Glucocorticoids are reported to be highly effective in controlling asthma and the first line therapy for chronic asthma (Barnes, 1995). However, there are concerns about the local and systemic side effects of inhaled glucocorticoids (Barnes *et al.*, 1993). The most common local side effect is dysphonia, which has previously been demonstrated to occur in 50% of patients that were receiving high dose therapy (Willey *et al.*, 1982). Long term systemic side effects of inhaled glucocorticoids include growth stunting in children and

the development of osteoporosis in adults (Barnes, 1995). Another major concern includes the increasing problem of glucocorticoid resistance (Barnes *et al.*, 1995).

Many of the existing therapies are targeted towards the inflammatory process that is associated with asthma rather than the inherently fragile bronchial epithelium. FXIII deficiencies have been described in chronic venous leg ulcer disease (Gemmati *et al.*, 2004) and inflammatory conditions of the gut, including ulcerative colitis (Pihusch *et al.*, 2002; Seitz *et al.*, 1994). Moreover, a study has demonstrated that cutaneous wound healing is impared in FXIII-deficient mice and that this may be overcome by treatment with FXIII (Inbal *et al.*, 2005). Topical application of FXIII, namely Fibrogammin® represents a relatively new concept in the treatment of FXIII-deficient diseases such ulcerative leg disease and has been successfully used to enhance wound healing of leg ulcers in patients with this disease (Herouy *et al.*, 2000; Wozniak *et al.*, 1999).

Given that the release of local coagulation factors have proven to be essential to normal bronchial epithelial repair, it would be interesting to compare the expression of coagulation factors between primary HBE cells of normal and asthmatic individuals. It is possible that FXIII may be deficient in asthma. The deficiency may not necessarily be inherited, but acquired. Firstly, FXIII is inactivated by nitric oxide, which is upregulated in severe asthma (Catani *et al.*, 1998). Moreover, neutrophil elastase is abundant in severe asthma and this enzyme has been reported to cleave FXIII. In the event that FXIII was demonstrated to be deficient in asthmatic subjects, it is conceivable that FXIII could be used in an inhalable form to overcome the deficiency and to promote epithelial repair. One concern associated with FXIII treatment is the possibility of absorption into the blood circulation leading to risks of thrombus generation. This may be overcome by combined therapy with heparin, the anti-thrombotic. The additional benefit of heparin is that it has anti-inflammatory properties and there is clinical evidence that heparin treatment provides benefit in asthma (Tyrrell *et al.*, 1999).

Elucidation of the mechanisms involved in normal bronchial epithelial repair may lead to a better understanding of the defective repair that is seen in the asthmatic airway. This could potentially lead to novel therapeutic strategies for the treatment of asthma.

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